6

New Knowledge

The Mechanisms of the Cytoplasm

> This continuous body of knowledge, which should be properly named cellular and molecular biology, could be compared to a bridge which, like its equivalents in civil engineering, has two bridgeheads: one in traditional anatomical-morphological sciences and the other in equally traditional biochemistry. The cautious and careful have stayed close to the bridgeheads because the area around them had been consolidated over centuries by the work of their predecessors. The bold and venturesome have ventured on the bridge itself from both directions, because they believed that there was where the action was going to be. . . . As in the old Latin proverb, fortune favored the bold: the bridge proved to be strong enough to support the intense occasionally frantic activity of whole armies of explorers.
>
> (Palade, 1987, pp. 112–13)

In the 1950s and 1960s the initial ventures into the terra incognita between classical cytology and biochemistry developed into the robust bridge Palade identified in the above quotation.[1] In large part this involved building on the localization of cellular energetics in the mitrochondria, and of protein synthesis in the microsomes, that had been established in the 1940s by decomposing these organelles and figuring out the operations associated with their parts. I will focus principally on these developments, but in the 1950s investigators identified the function of two other organelles – the Golgi apparatus and

[1] For Palade this was not just a perspective adopted in retrospect. Already in 1956 he commented on the integration of morphological and biochemical research: "The ample information obtained in each of these two fields has stimulated research in the other, with the result that a number of cell components have acquired a new biochemical and physiological significance. The concept of functional differentiation among cell organs has been more firmly established, and the previously sharp boundary between cell morphology and cell physiology and biochemistry has, to a large extent, faded away" (Palade, 1956a, pp. 186–7).

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the lysosome – and established research programs to determine how they performed their functions. In the final part of the chapter, therefore, I will provide a brief account of this research as well.

As I will show, the research extended far beyond the Rockefeller laboratory that provided the focus for most of the research described in the previous chapter. Nonetheless, it continued to play a central role, although the shape of the laboratory changed dramatically. In 1949 Claude accepted an invitation to direct the Jules Bordet Institute at the Université Libre de Bruxelles and left Rockefeller. In 1950 Murphy reached the mandatory retirement age (he died later the same year). At the Rockefeller Institute the usual procedure was to close a laboratory after the departure of a senior laboratory director (Member). However, in this instance, presumably in recognition of the pathbreaking work of the junior researchers and the investment in an electron microscope, Gasser took the unusual action of retaining Porter and Palade and promoting Porter to Associate Member and director of the laboratory. The characterization of the laboratory that year in the *Annual Report* reads, "During the period covered by this report studies on cell fine structures and related problems have been continued." (p. 143). The newly constituted Laboratory of Cytology moved to the basement of Theobald Smith Hall, where both the RCA EMU microscope that had been bought by the Rockefeller Foundation and a new RCA EMU-2A were installed.

Initially the group working with Porter and Palade was quite small. George Pappas spent two years as an Eli Lilly postdoctoral fellow. Maria Rudzinska, a protozoologist, worked with Porter. Sanford Palay and Don Fawcett, already faculty members at Yale and Harvard, respectively, spent considerable time visiting the laboratory. In 1955 Philip Siekevitz, a biochemist, joined the laboratory. In the middle 1950s the Rockefeller Institute was transformed from an exclusively research-oriented institution into a graduate university. The laboratories, previously staffed principally by scientists and postdoctoral researchers, now served as training centers for graduate students as well. Among the first graduate students in the laboratory were Mary Bonneville, Howard Rasmussen, Aaron Shatkin, Lee Peachey, and Peter Satir.[2]

[2] Peter Satir (Interview, 29 November 1995, Albert Einstein School of Medicine) related the unusual nature of the application process at Rockefeller in its first years as a university. President Bronk solicited nominations for graduate students from the top institutions in the country and interviewed applicants himself before directing them to the appropriate laboratories. According to Satir, Porter and Palade had been among the less eager investigators to make the transition to a graduate institution, but were exceptional in the support they provided to graduate students once they accepted them into the laboratory.

1. THE MITOCHONDRION

Biochemists Confront Particulate Structure: Mitochondrial Enzyme Systems

During the same period in which the Rockefeller researchers were situating succinoxidase and other oxidative enzymes in the mitochondrion and Claude was identifying its role as the power plant of the cell, numerous biochemists were following up on the thread from Keilin and Hartree's (1944) demonstration that they could not eliminate cell particulates from extract preparations capable of performing oxidative phosphorylation. Keilin and Hartree (1949) themselves interpreted this as indicating that the respiratory function was connected with the physico-chemical structure of the cell.[3] For most biochemists, however, the involvement of cell particulates was, as Lehninger put it, "a nuisance" (1951).[4] Their goal was to work out the purely chemical steps in oxidative phosphorylation in a manner comparable to that already provided for glycolysis.

As discussed in Chapter 3, by the 1940s there was a good understanding of the major operations in the three metabolic mechanisms that worked in sequence to oxidize carbohydrates – glycolysis, the citric acid (Krebs) cycle, and the electron transport chain (see Figure 3.16). After the investigations of Kalckar (1939) and Lipmann (1939), it was recognized that oxidative metabolism was linked to the storage of energy via ATP formation. In a theoretical paper, Lippman (1941) characterized the phosphate bonds in ATP as *energy-rich bonds* and introduced the symbol ~P for these; later they were more commonly referred to as *high-energy* bonds. The process by which energy was stored in such bonds was understood earliest (and turned out to be simplest) for the first two mechanisms, glycolysis and the citric acid cycle. As detailed in Chapter 3, in glycolysis the initial substrate (glucose) and the penultimate product (pyruvic acid) do not contain phosphates, but the various intermediates do. Phosphates are added at three different steps in the process and in later steps are transferred to ADP, yielding energy-rich ATP. This process later became known as *substrate-level phosphorylation*.

Knowledge of the process by which phosphorylation was coupled to the third oxidative mechanism in the sequence, the electron transport chain, was

[3] Keilin and Hartree (1949) themselves proposed that the particulate nature of their preparation facilitated respiration by assuring the mutual accessibility of the different enzymes encapsulated within each particle. Cleland and Slater (1953) determined that the Keilin and Hartree preparation included membranes from mitochondria.

[4] See also Lehninger (1964): "It was a part of the *Zeitgeist* that particles were a nuisance and stood in the way of purification of the respiratory enzymes" (p. 6).

more elusive and took much longer to achieve. One early constraint came from the work of Ochoa, who established that up to three molecules of ATP were formed per oxygen atom consumed. This indicated that the reactions forming ATP must occur at multiple steps along the electron transport chain. But what were these reactions? Lippman (1946) proposed that ATP synthesis along the electron transport chain would follow a scheme similar to that already known for glycolysis. He presented it in abstract form as a sequence of two reactions (the formulae above the line) achieving the overall effect of adding a phosphate bond to ADP to yield ATP (the summary formula below the line):

$$\mathrm{AH_2 + B + P_i \rightleftharpoons A \sim P + BH_2}$$
$$\underline{\mathrm{A \sim P + ADP \rightleftharpoons A + ATP}}$$
$$\mathrm{AH_2 + B + ADP + P_i \rightleftharpoons A + BH_2 + ATP.}$$

In these formulae, A denotes a substrate which is oxidized in the first reaction coupled with the reduction of another substrate, B. (A and B may be successive cytochromes in the electron transport chain, for example.) As A is oxidized it forms a high-energy bond with phosphate. In the second reaction, ATP is synthesized via the transfer of this bond to ATP.

This scheme was elaborated when it was discovered that the first of the two ATP-producing steps in glycolysis was more complicated than originally thought. The intermediate first gained energy as it was oxidized; only thereafter did it provide the energy for adding phosphate to ADP, yielding ATP. This led E. C. (Bill) Slater (1953) to revise Lipmann's proposed scheme for ATP formation coupled to the electron transport chain. He proposed that an additional compound, C, first formed a high-energy bond with A. The energy from that bond then facilitated the uptake of phosphate into ATP:

$$\mathrm{AH_2 + B + C \rightleftharpoons A \sim C + BH_2}$$
$$\underline{\mathrm{A \sim C + ADP + P_i \rightleftharpoons A + C \pm ATP}}$$
$$\mathrm{AH_2 + B + ADP + P_i \rightleftharpoons A + BH_2 + ATP.}$$

This version of the scheme set the agenda for many biochemists for the next twenty years – the race was on to identify C, the hypothesized intermediate. The search turned out to be futile, though, as no such intermediate exists.[5]

[5] Douglas Allchin has offered a detailed account of the quest for the nonexistent intermediate as well as lessons learned (1996; 1997). More recently he developed the idea that, although the search for a chemical intermediate could not succeed, other important results came out of the attempt. In particular, Allchin (2002) analyzed how Paul Boyer's research led to a number of discoveries, such as phosphohistidine, which figured as an intermediate not in oxidative phosphorylation, as

Phosphorylation along the electron transport chain has a very different explanation, as first outlined in the chemiosmotic hypothesis of Peter Mitchell (1961; 1966). Now called *oxidative phosphorylation*, this process turned out to depend upon the presence and structure of the inner membrane of the mitochondrion. The energy used to drive oxidative phosphorylation is stored, not in a chemical intermediate, but rather in a proton gradient across the membrane. With the eventual acceptance of this hypothesis, there no longer was any need to find the hypothesized chemical intermediate. Much more relevant were discoveries concerning the inner structure of the mitochondrion and the dependence of certain biochemical processes on that structure. These developments resulted from the interaction of biochemists and morphologists during the 1950s, which will be the focus of this section.

While Claude, Hogeboom, and their colleagues were making progress at the Rockefeller Institute, at the University of Chicago Albert Lehninger and his graduate student Eugene Kennedy were also conducting biochemical studies on particulate structures fractionated from rat liver. They, however, targeted the oxidation of fatty acids rather than carbohydrates. In ordinary liver preparations, a complex enzyme system catalyzes oxidation of the fatty acid octanoate (octanoic acid) via two possible pathways, one producing ketone bodies such as acetoacetate (acetoacetic acid) and the other proceeding through the citric acid cycle and respiratory chain, producing CO_2 and consuming oxygen to generate H_2O. Prior to Hogeboom et al.'s introduction of isotonic sucrose solution, Schneider, who was then still a graduate student at Wisconsin, had collaborated with Lehninger in testing a mitochondrial fraction of liver for evidence of fatty acid oxidation. Schneider tried supplying both water and saline suspensions of the mitochondrial fraction with octanoate and necessary supplementary substances (KCl, $MgSO_4$, a "sparking" Krebs intermediate, cytochrome *c*, phosphate buffer, ATP), but found no significant oxidation. Instead, he found oxidation occurring primarily in a fraction containing nuclei, erythrocytes, and some intact cells. After Hogeboom et al.'s (1948) paper, Lehninger set Kennedy to repeating this earlier work. Kennedy showed that with 0.88 *M* sucrose as the fractionation medium, octonoate oxidation occurred in the mitochondrial fraction (Kennedy & Lehninger, 1949).

previously hypothesized, but rather in substrate phosphorylation. Likewise, Boyer formulated an alternative mechanism of oxidative phosphorylation involving conformational changes that he initially advanced against both the chemical intermediate view and Mitchell's chemiosmotic view. He later recognized that his proposal was in fact compatible with Mitchell's account if he limited its scope to the actual synthesis of ATP. These efforts ultimately brought Boyer a Nobel Prize, even though his success did not lie in identifying the central mechanism of oxidative phosphorylation.

Lehninger (1951, p. 7) concluded from these investigations that "mitochondria contain a complete complement of the individual enzymes of the fatty acid oxidase system and in such amount that they could easily account for the known rates of oxidation in the intact cell."[6] Lehninger and Kennedy also found that mitochondria contain all the needed enzymes for oxidizing intermediates of the citric acid cycle, although they could not rule out the possibility that other fractions might also contain some of these enzymes. They also made an important negative finding – the reactions of glycolysis were catalyzed by the supernatant, not the mitochondrial fraction or other fractions with insoluble cell components. They concluded that glycolysis occurred in the aqueous cytosol of the cell's cytoplasm. Its product, pyruvic acid, would therefore need to be transported into the mitochondrion to join fatty acid products in the common pathway of the citric acid cycle.

In further research carried out with another graduate student, Morris Friedkin, Lehninger established, using labeled P^{32}, that isolated mitochondria synthesized ATP (Friedkin & Lehninger, 1949). In these initial studies, though, the rate of ATP formation was very slow. Lehninger (1951) discovered that when DPNH (NADH) was added to the mitochondrial preparation, it did not penetrate the mitochondrial body. He found that DPNH could enter the mitochondrion if he placed the preparation into a hypotonic KCl, sucrose, or distilled water medium for a short period before restoring isotonicity. Under these circumstances, oxidative phosphorylation occurred robustly and, in accordance with what Ochoa had found for tissue extracts, producing approximately three ATP molecules per atom of oxygen consumed.

A distinctive feature of Lehninger's theoretical outlook was that he interpreted difficulties in developing biochemical preparations for studying oxidative phosphorylation as clues to the importance of mitochondrial structure for those processes. This applied not only to the difficulty in getting DPNH to enter the mitochondria, but also to the fact that oxidative metabolism could only occur in preparations in which cell particulates remained. For him the failure to extract the responsible enzymes for oxidative metabolism when

[6] Noting the need to supply metabolites such as malate (malic acid), $MgSO_4$, cytochrome *c*, KCl, and ATP to maintain the reaction, Kennedy and Lehninger concluded, "Although the mitochondria appear to be the major site of these activities, it would appear from our examination *in vitro* that these bodies are not completely autonomous with respect to their respiratory behavior, since they must be supplemented with certain cofactors such as adenosine triphosphate and Mg++. It appears likely that in the cell there is a rapid interchange of these factors, substrates, and inorganic phosphate between the cytoplasm and the mitochondria. It also would appear that these bodies are dependent on the cytoplasm for certain preparatory metabolic activities such as glycolysis, since, as our data show, they are almost completely lacking in glycolytic activity" (1949, pp. 970–1).

liver tissue was ground in the Waring blender indicated that the mitochondrion was not merely a "sac containing a solution of soluble oxidative enzymes but . . . an organized structure with an insoluble matrix in which are embedded the individual catalytically active proteins making up the complex enzymatic machinery of oxidation and phosphorylation" (Lehninger, 1951, p. 12). Further influenced by the difficulty of accounting for the rates of oxidative phosphorylation by diffusion of intermediates, Lehninger advanced a bold proposal: "It would appear that these carrier proteins must be fixed in space so that hemes are juxtaposed, increasing the probability of fruitful collision, or that special mechanisms must exist to allow passage of electrons through the protein moieties" (p. 12).[7] Such a proposal departed radically from the traditional biochemical focus on soluble enzymes and helped set the stage for linking biochemical processes with cell structures.

David Green was another biochemist who responded to the difficulty of isolating oxidative enzymes by advancing the idea that the enzymes involved in oxidative phosphorylation constitute an organized system. Green had established his reputation through empirical work purifying and characterizing enzymes involved in cellular respiration while working with Malcolm Dixon at Cambridge in the 1930s (Green, 1936a; Green, 1936b; Green & Brosteaux, 1936; Green, Dewan, & Leloir, 1937; Green & Dixon, 1934) and by the publication of his 1940 book, *Mechanisms of Biological Oxidations*, which provided many in North America with their primary introduction to enzyme chemistry.[8] In 1948 he advanced the immediately controversial claim that the

[7] The next step in Lehninger's program was to determine where along the electron transport chain ATP synthesis occurs. He attempted to study the reactions starting with ascorbate (ascorbic acid, a nonenzymatic reductant of cytochrome *c*), and confronted the same difficulty – only when preparations were pretreated was the electron transport accompanied by phosophorylation of ADP. But with pretreatment, Lehninger was able to establish that one phosphorylating event occurred between cytochrome *c* and oxygen (see Lehninger, 1954). This implied that the other two phosphorylating events must occur earlier in the chain. Slater (1950) had previously found evidence for at least one phosphorylation earlier in the chain by using α-ketoglutarate (α-ketoglutaric acid) as the hydrogen donor and ferricytochrome *c* as the acceptor, and the question of exactly where in the electron transport chain the coupling with phosphorylation occurred remained a focus of inquiry throughout the 1950s. A major technique for approaching the problem was developed by Chance and Williams (1956). They used spectrography to determine the oxidation state of intermediates and, employing various inhibitors to impede the overall reaction and then adding ADP, they were able to identify zones in which each phosphorylation occurred.

[8] Green returned to the United States at the beginning of World War II and after a short period at Harvard, was appointed in 1941 assistant professor of biochemistry in the Department of Medicine at the Columbia College of Physicians and Surgeons in New York City. While he was in the midst of formulating the cyclophorase concept, he was approached by the University of Wisconsin, already a top institution in biochemistry, to become a founding member of the Institute for Enzyme Research at the University of Wisconsin. Green recruited an extremely

primary mechanisms of aerobic respiration (fatty acid oxidation, citric acid cycle, respiratory chain, and oxidative phosphorylation) were carried out by a single, physically structured system of enzymes that he called the *cyclophorase system* (Green, Loomis, & Auerbach, 1948). His primary evidence that these enzymes constituted a structured system was his failure to isolate the enzyme catalyzing pyruvic to acetic acid using cell fractionation techniques. Instead, his preparation from rabbit kidney, which involved homogenation with potassium chloride using alkali to neutralize the acid that formed, followed by multiple resuspensions in saline and centrifugation, metabolized pyruvic acid all the way to carbon dioxide and water. By referring to a cyclophorase *system*, he meant to contrast the enzymes involved in aerobic respiration with those involved in other biochemical processes such as glycolysis, purine synthesis, and the pentose and urea cycles. In those cases, the enzymes can be isolated and an operative system reconstituted from the isolated components. He explained the term *cyclophorase* as "literally meaning the system of enzymes carrying through the (citric acid) cycle" (Green, 1951b, p. 17). Green acknowledged that the ending "ase" is usually applied to individual enzymes, but cited precedent for his extension to a "team of enzymes":

> Keilin and his school have been referring for more than two decades to the succinic oxidase and cytochrome oxidase systems. Neither the one nor the other represents a single enzyme. They represent a considerable group of enzymes all of which are associated with the same particulate elements. (1951b, pp. 17–18)

Green conceptualized the cyclophorase system as involving a precise physical arrangement that would facilitate cooperative action between spatially proximal enzymes. He also maintained that this arrangement would enable the components to behave in ways they could not otherwise: "the chemical organization by which the many constituent enzymes are integrated confers properties on the various enzymes which they may not necessarily enjoy when separated from the complex and isolated as single enzymes" (p. 18). Although he initially claimed that the cyclophorase system represented a newly discovered constituent of the cell,[9] after he learned of Lehninger's work Green accepted

talented team of researchers to the Institute who helped identify many of the critical aspects of both fatty-acid metabolism and oxidative phosphorylation. His own research, however, became increasingly suspect (his preparations of his cyclophorase system were contaminated with many other cell components) and his theorizing less grounded in experimental evidence than other researchers thought appropriate.

[9] Both Van Potter (Interview, 6 November 1987, Madison) and Helmut Beinert (Interview, 5 November 1987, Milwaukee) noted that Green had to be convinced that his cyclophorase system was linked to the mitochondrion.

the Rockefeller group's linkage of these enzymes with the mitochondrion. He later wrote, "The mitochondrion and the cyclophorase system thus turned out to be the structural and functional sides of the same unit" (Green, 1957–8, p. 178). He nonetheless advocated using the name "cyclophorase system" "for the functional attributes of the same entity" (1951b, p. 19, n. 2). Green credited Harman (1950a), who was working with him at the Enzyme Institute, with establishing the proportionality of cyclophorase activity and the presence and number of mitochondria.

An important aspect of Green's conception of the cyclophorase system was that it not only linked together the enzymes but also bound them to the coenzymes that figured in the reactions. Washing the preparation would remove the coenzymes and, as well, most of the NAD, NADP, FAD, and ATP in the cell that was normally bound in the cyclophorase system. Green proposed further that a coenzyme was bound as a *prosthetic group* to the protein component of an enzyme, which he referred to as the *apoenzyme*, and that when the two were split, the enzyme was modified. Green suggested that such an arrangement was most efficient in that it required only one coenzyme molecule per enzyme molecule, whereas if they were dissociated and relied on random processes such as diffusion to encounter each other, many times more coenzyme molecules would be required. Green did note a serious problem posed by binding of the coenzyme to the enzyme:

> . . . pyridinenucleotide must be capable not only of being reducible by the substrate of the oxidase with which the former is combined, but also in its reduced form has to interact with the flavin prosthetic group of diaphorase – the enzyme which catalyses the oxidation of dihydropyridinenucleotide by one of the cytochrome components. When the pyridinenucleotide is free as in the case of the classical, soluble systems, this sequence of reactions poses no difficulty. The coenzyme is free to shuttle back and forth . . . In the cyclophorase system with bound pyridinenucleotide, the extent of shifting back and forth is severely limited. Some mechanism must be invoked to explain how a coenzyme fixed in a rigid structure would be capable of interacting with a variety of systems. (1951a, p. 429)

Most biochemists rejected Green's cyclophorase proposal as an excessively speculative response to the difficulty of rendering the enzymes of oxidative metabolism soluble and isolating them. Nonetheless, like Lehninger, those biochemists working on oxidative phosphorylation in the early 1950s came to recognize, if only as a nuisance factor, that the enzymes of oxidative phosphorylation were localized in the mitochondrion and in some intimate way connected with mitochondrial membranes. The question was how. The next

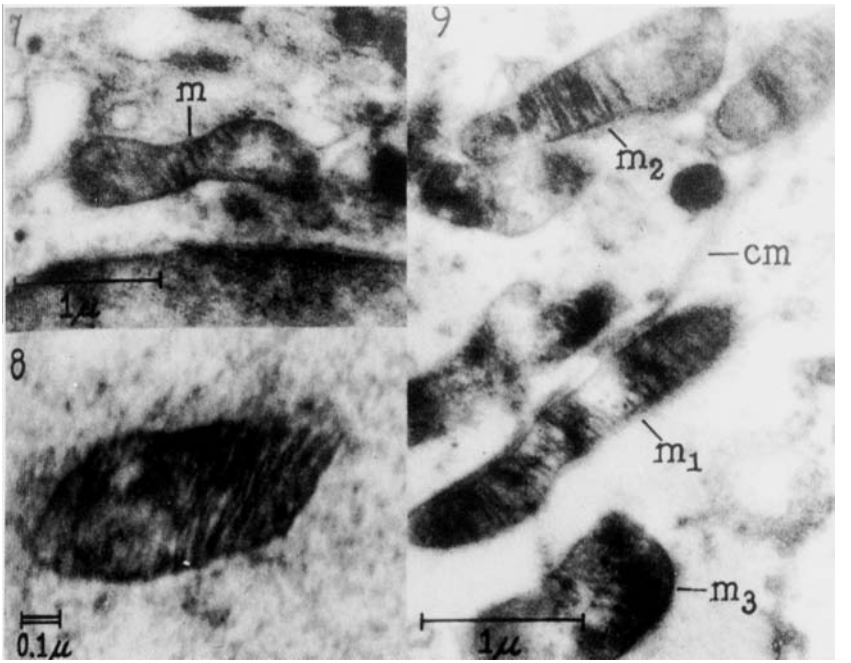

Figure 6.1. Three of Palade's 1952 micrographs revealing what he called *cristae* and construed as infoldings of the mitochondrial membrane. Reproduced from G. E. Palade (1952a), The fine structure of mitochondria, *Anatomical Record, 114*, 427–51, Plate 3, Figures 7–9, p. 449, with permission of John Wiley.

important clue stemmed from a discovery of additional structure in the mitochondrion made possible by improvements in electron microscopy.

More Structure: The Discovery of the Cristae of the Mitochondrion

As noted in Chapter 4, Palade (1952b) conducted a comparative investigation in the early 1950s that resulted in a new buffered osmium fixative. In a study using it as well as Porter's new microtome to cut thin slices, Palade announced the discovery of a "system of parallel, regularly spaced ridges that protrude from the inside surface of the membrane towards the interior" (1952a, p. 428).[10] They are visible in the micrographs in Figure 6.1. Palade

[10] In the same study Palade found clear evidence for the mitochondrial membrane, although he initially took it to be a single membrane. The existence of a mitochondrial membrane had been previously defended both on the basis of electron microscopy of isolated mitochondria and on the basis of biochemical findings about the soluble character of chemical compounds that are retained in the mitochondria, but it was also questioned by others (Harman, 1950b; Huennekens & Green, 1950).

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noted that the ridges appeared most clearly when sections were cut longitudinally through each mitochondrion and that they were oriented "more or less perpendicular to the long axis of the mitochondrion" (p. 432). He observed that the ridges have a "trilaminar structure" with "a central layer 8 to 10 mμ thick" surrounded by two thinner and denser layers. He called these ridges or protrusions "cristae mitochondriales" (p. 433) and, although noting that their appearance varied between cell types, concluded that they were likely to be regular features of mitochondria. He even suggested that they could serve as a "criterion for the identification of mitochondria in electron microscopy where the characteristic staining reactions of these organelles are of no avail" (p. 438).

By differentiating the cristae from the fluid matrix within the interior of the mitochondrion, Palade offered a structural decomposition that raised the prospect of advancing the mechanistic account of mitochondrial function by localizing different biochemical operations in different structural components. Palade himself put forward the suggestion that the aerobic oxidation reactions were directly tied to the structure of the mitochondrion, especially the cristae:

> It is well established that isolated mitochondria are able to carry out *in vitro* complicated oxidative processes that imply the action of a considerable number of enzymes. As these oxidations are apparently well co-ordinated and, in addition are dependent on the morphological integrity of the mitochondria . . . , it has been postulated that the enzymes involved in such processes are maintained in a 'definite spatial relationship' (Schneider & Hogeboom, '51) inside the organelles. It may be assumed that they are arranged in the proper order in linear series or chains – a disposition comparable in design and efficiency to an industrial assembly line. Such enzymatic chains have to be built at least partially in the solid framework of the mitochondrion because some of the component enzymes, namely succinic acid dehydrogenase (succinoxydase) and cytochrome *c* oxydase, are known to be insoluble and structure-bound. If we integrate the present morphological information with what is known from the general behavior of the mitochondria (e.g., their flexibility, response to variations in osmotic pressure and results obtained by centrifuging disintegrated organelles), it may reasonably be assumed that the mitochondrial matrix is fluid and that the membrane and the ridges represent the solid framework. In the present state of our knowledge, the internal ridges of the mitochondria appear as the most probable location for the postulated enzyme chains. (pp. 438–9)[11]

[11] In a footnote, Palade commented on Green's proposal of a cyclophorase system: "The work referred to deals with some dehydrogenases of the 'cyclophorase system,' a tissue residue

In a paper the following year Palade (1953) offered a number of arguments for localizing the enzyme systems in the cristae. First, he noted that a number of experiments, such as those of Lehninger, indicated that a substrate has to penetrate inside the mitochondrion before it is acted upon. Second, he pointed out that particles comparable in size to individual cristae and containing most of the succinoxidase systems of the mitochondria had been isolated from suspensions of disintegrated mitochondria (see below). Finally, he suggested that if the enzyme systems were located in the cristae rather than the outer membrane, they would be less exposed and protected from disruption.

A Competing Perspective on Mitochondrial Morphology

Although the Rockefeller laboratory of Porter and Palade played the central role in developing the new conceptions of cell structure and function discussed so far, there were competing laboratories, especially in Europe, which challenged several of their claims. The most vocal opponent was Fritiof Stig Sjöstrand, who in the 1950s established a major electron microscopy laboratory at the Karolinska Institute in Stockholm. As a medical and doctoral student at the Karolinska during World War II, Sjöstrand had begun working with an electron microscope built by Manne Siegbahn, a physicist at the Royal Swedish Academy of Sciences. Based on his early attempts to develop thin sections he had published micrographs of muscle in *Nature* in 1943 that were suggestive but provided little detail (Sjöstrand, 1943). In September 1947, in a meeting with R. R. Struthers, he (unsuccessfully) appealed to the Rockefeller Foundation for an electron microscope, noting that the only functioning microscope in Stockholm was in the Department of Histology. Struthers noted in his diary that Sjöstrand "appears more than usually intelligent and diligent and makes an excellent impression."

Supported by a Swedish State Research Council Fellowship, Sjöstrand spent the 1947–8 academic year at MIT working with Francis Schmitt on

lately identified as mitochondria. It is known (Schneider & Hogeboom, '51) that this system is actually a mixture of cell debris, nuclei, and mitochondria, a fact that renders more difficult the interpretation of the results mentioned. What happens in a 'cyclophorase system' does not necessarily take place exclusively in mitochondria" (p. 437). For his part, Green credits the electron micrographs of Palade and Sjöstrand with providing "independent confirmation of the organization deduced from functional considerations. These microscope studies readily disposed of the then current hypothesis that all the enzymes and coenzymes of the mitochondria were present as freely diffusible molecular species without any special organization in the fluid interior of the mitochondrial 'bag,' which was surrounding by a semipermeable membrane" (Green, 1957–8, p. 178).

the ultrastructure of the retinal rods.[12] There he further developed his abilities in electron microscopy. After returning to Sweden in 1948, Sjöstrand secured an appointment as permanent Docent in the Department of Anatomy at the Karolinska Institute, a position with limited teaching obligations that permitted him to devote most of his time to research. He set out to establish a laboratory for what he called *ultrastructure research* and in 1949 secured funding from the Alice and Kurt Wallenberg Foundation for an electron microscope (RCA EMU 2C). In the context of an application to the Rockefeller Foundation for additional equipment, he identified three components of his continuing research: the "structural basis of irritability" in sensory cells (the project Sjöstrand began at MIT), thin sectioning, and fixing cells for electron microscopy. The principal specimens for this work were the rods and cones of the guinea pig, especially a thin membrane which he thought was the primary part of the cell that was stimulated by light.

In 1949, as part of a trip to the Electron Microscope Society of America meetings in Washington, Sjöstrand visited the Rockefeller laboratory for a month and had access there to Claude's new microtome. Upon his return to Sweden, he developed his own microtome, one that employed an eccentrically located tissue mount revolving sixty times per minute that was advanced by a thermally expanding column behind the eccentric wheel. With this microtome, Sjöstrand claimed to be able to cut sections as thin as 70 Å on a regular basis. According to Porter,[13] Sjöstrand returned to Rockefeller for a month in 1952, where he learned of Palade's new buffered osmium fixative.

[12] Schmitt's impressions of Sjöstrand based on that year, reported in a letter to Gerald Pomerat of the Rockefeller Foundation on 26 July 1950, were certainly mixed. Schmitt said,

> I think there is little doubt that he is competent in [electron microscope research]. He is rather slow and sometimes appears phlegmatic, but this is probably illusory for he acquits himself well in discussions or debates, especially when his own work is in question.
>
> I am not sure that Sjöstrand himself will make any brilliant advances, but I do hope that his laboratory will become an active center for tissue fine structure work. Sjöstrand is well grounded in the field and will doubtless make substantial contributions, but I think his leadership among younger students of anatomy may pay even greater dividends. (Folder 1947, 1949–51, Box Karolinska Institutet, Molecular Biology, Series 800D, RG 2, Rockefeller Foundation Archives, RAC.)

Two years later Schmitt was more positive in his assessment of Sjöstrand's work at MIT. In a letter of 25 November 1952 to Ture Petrén, head of the Anatomy Institute in which Sjöstrand's laboratory was housed, Schmitt said, "I found that he is a sound scientist with the patience necessary to develop the complicated techniques required for the successful application of electron microscopy to the study of cell structure" (folder 1952–6, Box Karolinska Institutet, Molecular Biology, Series 800D, RG 2, Rockefeller Foundation Archives, RAC).

[13] Interview with Keith Porter, 1987, University of Maryland, Baltimore County. According to Porter, Sjöstrand also induced Porter's technician to return to Sweden with him, although she later returned to resume work with Porter.

Figure 6.2. Sjöstrand and Rhodin's micrograph of mitochondria in guinea pig retina revealing what they called *internal membranes* and construed as separate structures, discontinuous with the outer mitochondrial membrane and with each other. Reproduced from F. S. Sjöstrand and J. Rhodin (1953), The ultrastructure of the proximal convoluted tubules of the mouse kidney as revealed by high resolution electron microscopy, *Journal of Experimental Cell Research, 4*, 426–56, Figure 2b, p. 434, with permission of Elsevier.

That autumn he made a major splash at the Electron Microscope Society of America meetings by presenting new, very high resolution micrographs from guinea pig retina, later published in *Nature* (Sjöstrand, 1953b) that revealed Palade's cristae more clearly than Palade's own micrographs of the

period.[14] Similar micrographs appeared in a paper that was part of the doctoral dissertation of one of his students, Johannes Rhodin (Sjöstrand & Rhodin, 1953). One of these micrographs is reproduced in Figure 6.2. Sjöstrand and Rhodin proclaimed,

> This investigation has demonstrated an internal structure within the mitochondria, which, as far as we know has not been described before. The system of transversally orientated double membranes and the clear cut demonstration of a similar outer membrane around the mitochondria indicate a high degree of organization of these cell organelles. (p. 449)

He described the structure more fully in his paper with another student, Viggo Hanzon (Sjöstrand & Hanzon, 1954):

> In the interior of the mitochondria densely packed inner membranes or plates are seen mainly oriented perpendicularly to the long axis of the mitochondrion. The inner membranes also appear double edged. One end of the membranes is in contact with the outer surface membrane and the other end in most cases is free from this membrane. (p. 406)

Although acknowledging occasional contact, Sjöstrand went on to state, "There is with few exceptions no continuity observed between the central space in the inner and outer membranes" (p. 406).

Sjöstrand's interpretation of what his micrographs revealed about mitochondria differed with Palade's views on two major points.[15] First, he maintained that the mitochondrial membrane was comprised of two layers, not one. Second, he rejected Palade's claim that the cristae were infoldings of the mitochondrial membrane: "There are no indications that the inner membranes represent folds of a single edged surface membrane. The inner membranes are

[14] Sjöstrand's presentations at these meetings were a major boost to his reputation. His former mentor Schmitt, who had previously been cautious in his appraisals of Sjöstrand, commented, "We have been mildly skeptical of his claims that he can section to 200–500 Å consistently. This skepticism was completely removed at the Cleveland meetings of the Electron Microscope Society of America earlier this month. At these meetings he described his results on the pancreas cells, the convoluted tubule cells of the kidney, the retinal rods and cones, and the nerve myelin sheath. Any of these papers would have been a great credit to the most experienced authorities in the field. However, the combination of the four was easily the best work reported at the meetings – a real triumph for Sjöstrand." (Letter of 25 November 1952 to Ture Petrén, Head of the Anatomy Institute in which Sjöstrand's laboratory was housed, Folder 1952–6, Box Karolinska Institutet, Molecular Biology, Series 800D, RG 2, Rockefeller Foundation Archives, RAC.)

[15] Rasmussen (1995; 1997) offered a detailed account of the dispute between Palade and Sjöstrand in which he argued that differences in their interpretive styles, including how they related electron microscopy to biochemistry, were more important than differences in technique: "The structure of nature and the structure of the sciences were at stake simultaneously in the struggle over which interpretive method should be made standard, which practices 'proper'" (p. 151).

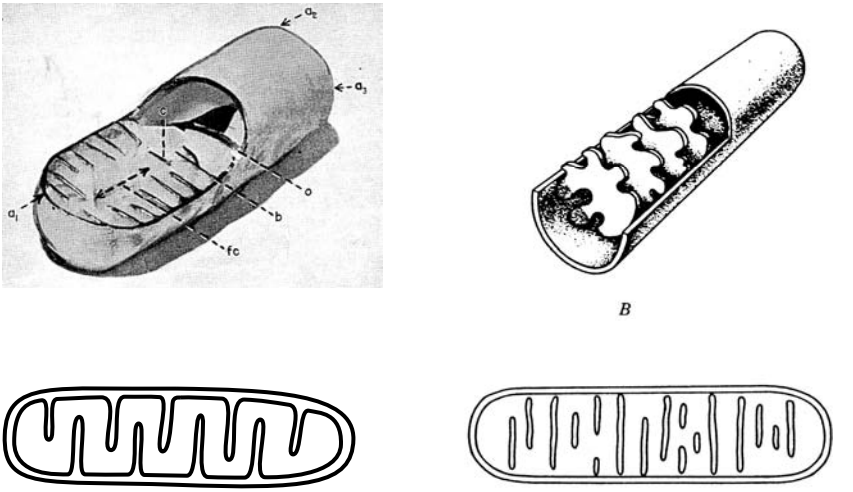

Figure 6.3. Contrasting interpretations of the internal structure of the mitochondrion as 3D models (top) and as 2D medial longitudinal sections (bottom). Palade's 3D model (left) is reproduced with permission from G. E. Palade (1953), An electron microscope study of mitochondrial structure, *Journal of Histochemistry and Cytochemistry, 1*, 188–211, p. 197. The 2D diagram is drawn by the author. Sjöstrand's 3D and 2D diagrams (right) are reproduced from F. S. Sjöstrand (1956), Electron microscopy of cells and tissues, in *Physical techniques in biological research*, G. Oster and A. W. Pollister, Eds. New York: Academic Press, pp. 241–98.

individual structures with only topographic relations to the outer membrane. Therefore the term, *cristae mitochondria*, is misleading" (p. 413).

The following April, at a symposium on the structure and chemistry of mitochondria at the Histochemical Society meetings in Chicago, Palade (1953) accepted Sjöstrand's claim that the mitochondrion was bounded by a double membrane,[16] and proposed that it was only the inner membrane which folded into the interior to form cristae. Sjöstrand remained opposed to the idea of infolding, arguing that the double-layered membranes traversing the mitochondrion were not attached to the (also double-layered) outer membrane. He also claimed that these double-layered traversing membranes went fully across the mitochondrion, whereas Palade proposed that there was an open channel extending through the interior of the mitochondrion, which he

[16] Sjöstrand himself referred not to two membranes but to a single double-layered membrane which he interpreted in terms of Danielli and Davson's sandwich model (see Figure 3.4). He held that the outer layers of proteins accounted for the two dark bands while the inner phospholipid layer accounted for the lighter area between them. Palade, not trying to provide a physical interpretation of membrane structure, simply interpreted each dark band as a separate membrane.

thought might serve for diffusion of substrates and products.[17] As shown in the top half of Figure 6.3, in 1953 both men produced 3D models – Palade a wax model and Sjöstrand a diagram – that illustrate the differences in how they conceived of these structures. Note especially the area in Palade's model labeled *fc* (for *central free channel*, later called the *mitochondrial matrix*). The two sketches in the bottom half of Figure 6.3 schematically illustrate a medial longitudinal section through each 3D model (discussed below).

The controversy between Sjöstrand and Palade was often personal and acrimonious.[18] Sjöstrand argued vociferously for the higher quality of his micrographs and for artifacts in Palade's micrographs. In particular, at the Third International Conference on Electron Microscopy, held in London in 1954, he proposed that the appearance of an open channel in the middle of mitochondria in Palade's micrographs was due to poor preservation as a result of delays in fixing the specimens (Sjöstrand, 1956b). These delays, he alleged, led to swelling during the postmortem interval before fixation was complete. To demonstrate the process he thought gave rise to Palade's micrographs, he prepared a series of micrographs at different intervals and claimed that the later micrographs showed increasing disruption of the internal membranes:

> Studies of post-mortem changes taking place within 15–30 to 45 minutes after death have shown that the mitochondria are changed very soon after death. They swell after the shutting off of the blood supply, the inner mitochondrial membranes appear fragmented and pulled apart leaving a more or less extensive central space free from inner membranes. It might be that such postmortem changes are responsible for the central space described by Palade. (Sjöstrand, 1956a, p. 463)

Sjöstrand maintained that a primary factor in generating high-resolution micrographs was keeping the tissue in as life-like a condition as possible. Thus, he injected fixative directly into living animals and as quickly as pos-

[17] In his 1952 paper Palade had commented, "In longitudinal sections that cut close to the mitochondrial membrane, the appearance of the lamellae might suggest that they are septa that traverse the mitochondrion from side to side. Oblique sections, however, indicate that the lamellae are actually ridges or folds protruding from the inside surface of the mitochondrial membrane towards the interior of the organelle without reaching the other side" (p. 432).

[18] At least in the eyes of Keith Porter, Sjöstrand's success was derivative from his and Palade's efforts. He complained to Pomerat that Sjöstrand failed to sufficiently acknowledge the assistance he had received. Yet, in his paper with Rhodin on the ultrastructure of mouse kidney tubules Sjöstrand says, "We feel very much indebted to Dr. K. Porter and to Dr. G. E. Pallade [employing the original spelling for Palade's name] for their kindness in giving the necessary information regarding the fixation technique used in the investigation. During the printing of this paper the paper by G. E. Pallade describing the fixation technique in *J. Exptl. Med.* **95**, 285 (1952) has become known to us" (Sjöstrand & Rhodin, 1953, p. 427, n. 1).

sible cut the tissue and immersed it in a phosphate-buffered saline solution maintained at salt and pH levels comparable to those found in living organisms.

In 1954 Sjöstrand presented a paper at the VIIIth Congress of Cell Biology in Leiden in which he set out to "survey the studies of the mitochondria structure that have been performed by Palade at the Rockefeller institute in New York and by our group at the Karolinska institutet in Stockholm" (1955a, p. 16). He presented only his own micrographs and began the paper by offering his interpretation of mitochondrial structure. But he then turned to the differences, noting first that "These discrepancies were more pronounced earlier but have been reduced with the improvement of the technique used by Palade" (Sjöstrand, 1955a, p. 19). Sjöstrand reiterates his contention that the inner membranes are not continuous with the membranes surrounding the mitochondrion and that there is no central space extending the length of the mitochondrion. He allowed that sometimes the membranes "do not form complete septa" so that there is communication between some of the compartments created by the inner membranes. But, he contended,

> The central space as depicted by Palade appears to us as a fixation artefact due to swelling of the mitochondria. Palade, seems not to have observed the susceptibility of the mitochondria to hypotonic media as the buffered osmium tetroxide solution originally recommended by him is strongly hypotonic. A similar swelling also occurs in a fairly rapid post mortem change and, therefore, appears deaper [sic] than 40–50μ below the surface of the tissue block. Palade's pictures show an appearance of the mitochondrion which is similar to the one we have observed as a result of post mortem changes. (p. 21)

In that paper Sjöstrand also took exception to proposals (such as Palade's, although he did not name Palade) linking the structural features of mitochondria to their biochemical function:

> What does this organization of the mitochondria mean? We may talk about the membranes as useful in realizing an orderly arrangement of the enzyme molecules to give fortunate spatial relations between enzymes taking part in chain reactions. We do not know, however, where the enzyme molecules are located and I think the speculations regarding the functional significance of these structures at this state [sic] may be reserved for very informal discussions or private contemplation. (p. 29)

Palade (1953), for his own part, explicitly credited Sjöstrand only for his determination that the mitochondrial membrane is a double membrane. Without specifically naming Sjöstrand, he offered a critical test between Sjöstrand's and his own proposals regarding the cristae. If they were true septa, he argued,

"they should appear as continuous, traversing bands in all longitudinal sections, irrespective of their position in relation to the axis of the organelle" (p. 205). However, if they are ridges that project only partway into the interior, then "they should appear as continuous, traversing bands, only in longitudinal sections cutting close to the mitochondrial membrane" (p. 205). In other sections "passing lengthwise through the middle of the organelles, the lamellae should show free ends partially outlining a central channel or cavity" (p. 205). Palade contended that his micrographs revealed the second pattern. Palade also appealed to sequential serial sections to establish the claim that the cristae are really ridges, showing that different ridges disappear as one moves from slice to slice. Palade also presented evidence that the cristae are actually infoldings of the inner mitochondrial membrane and that "the light space between the two mitochondrial membranes is found to have approximately the same width as the central light layer of the cristae, with which it appears to communicate freely" (p. 207). Later this was called the *intermembrane space*.

In a 1956 paper in which he provided frequent citations to Sjöstrand's work, Palade, after indicating that most of the knowledge of fine structure came from his and Sjöstrand's groups, drew out the contrasts and ended by taking as conciliatory a stance as possible:

> The structural details described are the same, but there are, as expected, certain differences in interpretation and nomenclature. For instance, the Swedish group presents the two membranes at the periphery of the mitochondrion as a single structural unit, as a "double-edged" membrane under the name "outer double membrane." The cristae are not recognized as infoldings but described as "individual structures with only topographical relations to the outer membrane." The term "inner double membranes" is used for their designation. The points in disagreement are decreasing in number, however. For instance, the existence of two dense lines at the periphery of mitochondrial profiles, revealed by Sjöstrand's work, was subsequently confirmed by us, and the cristae ("inner double membranes"), originally described as complete septa by the Swedish group, are now recognized as incomplete partitions, at least in some cases. (1956a, pp. 194–5)

The sketches in Figure 6.3 of medial longitudinal sections through the two men's models illustrate how each inferred a different internal structure from micrographs that were not sufficiently detailed to resolve the issue to everyone's satisfaction. Palade's interpretation (bottom left) yielded two aqueous areas, each of which is in communication with a large surface area on one side or the other of the convoluted inner membrane (whose projections into

the matrix were the cristae). One theme in Palade's paper was that these structural characteristics provided ample opportunity for the types of biochemical reactions identified in cell fractions. In contrast, Sjöstrand's interpretation (bottom right) yielded multiple pieces of inner membrane that sometimes touched the inner layer of the outer membrane but were structurally separate – they were discontinuous both with the outer membrane and with each other. In the type of cell Sjöstrand used for his earliest micrographs, exhibiting plate-shaped rather than puzzle-piece-shaped inner membranes in 3D, the inner membranes typically extended across the entire 2D section, touching at both ends – the "complete septa" referred to by Palade. Regardless of how completely the mitochondria were partitioned in Sjöstrand's account, the resulting topography would turn out to be inconsistent with an important biochemical account proposed in 1962 and eventually accepted (Mitchell's chemiosmotic hypothesis; see Figure 6.8).

Sjöstrand never capitulated, but the intense conflict soon abated as Palade's interpretation gained ascendancy (for details, see Rasmussen, 1997[19]). Just a few years later Bourne (1962) could write matter-of-factly, "after a little controversy, it was agreed that the inner of these two membranes was extended into the interior of the mitochondria, in some cases touching or almost touching the other side" (p. 59).

Biochemists Further Fractionate Mitochondria

The cristae offered a plausible locus for the biochemical mechanism of oxidative phosphorylation, which biochemists already recognized as membrane bound. But exactly how were the enzymes recognized as responsible for the different reactions bound in the membrane? As Rasmussen emphasized, one of the features of the Rockefeller approach was that it promoted a collaborative inquiry with biochemists in which techniques for studying mitochondria were complementary, not competitive: Biochemical reactions could be localized in particular parts of the cell via chemical analysis of fractions while

[19] In discussing the interaction between Palade and Sjöstrand at the Third International Conference on Electron Microscopy in London, England, in July 1954, Rasmussen focused on the different uses to which each put his micrographs. Sjöstrand stressed using micrographs to make quantitatively precise estimates of membrane sizes. Palade, on the other hand, emphasized the importance of drawing out the connections with findings from other techniques such cell fractionation. Thus, Rasmussen commented, "Sjöstrand wanted to interpret his micrographs purely visually, judging fixation by the criterion of orderliness and seeking greater knowledge of molecular structure through ever-better resolution, whereas Palade, who was involved in cell fractionation himself, wanted to test micrograph interpretations against experiments on fractions" (1997, p. 139).

the cell parts and their organization were determined by electron microscopy. Contrary to Sjöstrand, Palade did not think either approach could yet yield descriptions of cell parts at the molecular level.[20] Once it was established that the processes of aerobic respiration as a whole were localized in mitochondrial fractions, the natural extension of the research was to seek to localize different enzymes in different components of the mitochondrion. The obvious way to proceed was to apply fractionation again, decomposing the whole mitochondrial system into subfractions that could carry out some but not all the operations of oxidative phosphorylation. If this were successful, researchers could hope to localize these reactions in turn in parts of the mitochondrion that appeared in different fractions. Again Green and Lehninger were the leaders in deploying this strategy, joined subsequently by Efraim Racker.

Taking advantage of the availability of beef hearts from the nearby slaughterhouses in Wisconsin, Green developed a procedure for large-scale fractionation of mitochondria. The procedures he employed routinely damaged mitochondria, but had the advantage of yielding components with different behavior. First, he obtained a *light fraction* that lacked the capacity to synthesize ATP when oxidizing succinate and a *heavy fraction* that retained that capacity. Both fractions, though, phosphorylated ATP when other citric acid cycle substrates were supplied. Green further divided the light fraction (after treating it with 15% alcohol) into subfractions, one of which carried out electron transport but not oxidative phosphorylation (he referred to these as

[20] Rasmussen maintained that a fundamental difference between Palade and Sjöstrand involved their respective relations to biochemistry: "In the science Sjöstrand was trying to build, the electron microscope presumed a certain authority over the territory of biochemists, who were heavily invested in their 'slick' new ultracentrifuges but were in a very weak position to establish for themselves that the cell components they were isolating had not been drastically altered by cell homogenation and the lengthy centrifugation protocols. On the other hand, the Rockefeller way posits a partnership with the fractionation biochemist, and a set of more modest goals for the electron microscopist that prevents conflict with the biochemist partner: mere description of topology of and associations among components in the unfractionated, in situ cell. The Rockefeller cell biologists had a metier whose definition did not entail conflict with the established biochemistry departments at institutions where electron microscopists were finding work in the later 1950s and 1960s" (1997, p. 148). Rasmussen developed this argument as part of a sociological explanation for the greater success of the Rockefeller approach and acceptance of the Rockefeller results. As discussed in the previous chapter, the technique of fractionation itself originated with the efforts of Bensley and especially Claude to link cell structures to biochemical operations. The Rockefeller researchers, including Palade, continued to utilize fractionation as a primary tool in their own research (see the discussion in Part 2 of this chapter of Palade and Siekevitz's collaboration on the endoplasmic reticulum). Thus, another way of viewing the Rockefeller approach is to trace its development as an internally motivated program that, as a happy side effect, minimized territorial conflicts. This differs from Rasmussen's perspective in that it subordinates sociological factors to scientific ones.

electron transport particles or ETP). Another fraction supported phosphorylation when oxidizing compounds other than succinate (he termed these *phosphorylating electron transport particles* or PETP). To understand the genesis of these particles, Green collaborated with electron microscopist Hans Ris.[21] The resulting micrographs revealed open fragments of cristae in the PETP and less functional closed fragments of cristae in the ETP particles (Green, 1957–8; Ziegler et al., 1958). This supported Palade's suggestion that the processes of oxidative phosphorylation were localized in the cristae.

Youssef Hatefi, working in Green's laboratory, developed evidence for grouping the various substances involved in the electron transport chain into four complexes:

(I) an NADH-ubiquinone reductase complex which included FMN and nonheme iron
(II) a succinate-ubiquinone reductase complex which included FAD and nonheme iron
(III) a ubiquinol-cytochrome *c* reductase complex which included cytochromes *b* and c_1, and a nonheme iron protein; and
(IV) a cytochrome *c* oxidase complex which included cytochrome *a* and copper.

Thereafter four of Green's collaborators, Hatefi, Haavik, Fowler, and Griffiths (1962), succeeded in reconstituting two systems: one capable of oxidizing NADH to carbon dioxide and water by combining complexes I, III, and IV, and another capable of oxidizing succinate to carbon dioxide and water by combining complexes II, III, and IV. Both reconstitutions revealed particulate structures, suggesting that the respiratory chain was formed into a fixed assembly electron transfer system (one in which the molecules were in advantageous spatial relations for passing electrons sequentially from molecule to molecule).

[21] Ris did his graduate work on mitotic division with Franz Schrader in zoology at Columbia. Subsequently, he spent a number of years working in Mirsky's laboratory at Rockefeller. Mirsky was a biochemist by background and Ris characterizes him as teaching Ris biochemistry while Ris provided cytological understanding of the structure of chromosomes as well as procedures for isolating them. Together they published a number of papers revealing, for example, the role of proteins in providing structure to chromosomes (Mirsky & Ris, 1951) and generating quantitative measures of DNA content in cell nuclei (Ris & Mirsky, 1949). Ris moved to the University of Wisconsin in 1949 and shortly thereafter began to explore the potential of electron microscopy for studying chromosomes, a project that did not fully bear fruit until the development of high-voltage electron microscopes in the late 1960s. Ris found Green's lack of sensitivity to cytological structure frustrating and soon suggested that Green work instead with Fernández-Morán at the University of Chicago (Interview, 6 November 1987, Madison).

Together with Cecil Cooper and other colleagues, Lehninger also pursued a project of "making morphologically less organized preparations from rat liver mitochondria with which the enzymatic details of oxidative phosphorylation could be more directly studied, with the ultimate goal of resolving the mechanisms of oxidative phosphorylation by enzyme separation and reconstruction approaches" (Lehninger et al., 1958, p. 450). The last clause indicates Lehninger's goal of establishing the mechanisms of oxidative phosphorylation using the traditional biochemical approaches of isolating responsible enzymes and then putting them together again into a system that performs the reaction.

The strategy Lehninger and his collaborators employed for decomposing the mechanism was to treat isolated mitochondria with digitonin to gently break down membrane lipids, which they reported caused the "virtual dissolution" of the mitochondrion, "leaving a turbid brown solution" (p. 450). They subjected this solution to centrifugation at 50,000 *g* for 25 minutes, removed the supernatant fluid, which contained a gelatinous material, and centrifuged it at 100,000 *g* for another 25 minutes. This yielded "phosphorylating membrane fragments" (p. 450), which did not catalyze most of the reactions of the citric acid cycle and generated ATP only when D-β-hydroxybutyrate or succinate (but not pyruvic acid or some other citric acid cycle intermediate) was supplied. Electron transport was evidenced by oxygen uptake, and oxidative phosphorylation by ATP synthesis (using radioactive phosphate as a tracer). Although the efficiency of the reactions was less than for intact mitochondria, they claimed that ATP synthesis did occur at all three sites along the electron transport chain. Most of the typical chemical agents that decoupled respiration from phosphorylation, such as 2,4-dinotrophenol, had the same effect on the particles, but Ca^{2+} and thyroxine did not. Appealing to electron micrographs, Lehninger and colleagues identified the particles as arising largely from the cristae.

From two facts – that the citric acid cycle enzymes could be separated in solution and that the membrane fragments isolated by Lehninger did not catalyze the reactions of that cycle – investigators could conclude that the citric acid cycle occurred in the matrix of the mitochondrion while electron transport and oxidative phosphorylation occurred in the cristae of the inner mitochondrial membrane, as Palade had suggested. The earlier determination that glycolysis occurred in the cytosol permitted localizing the three major biochemical mechanisms of cellular respiration (as detailed in Figure 3.16) in three different parts of the cell, as illustrated in Figure 6.4.

From the fact that sonic vibrations prior to centrifugation greatly reduced the sedimentation rate but not the efficacy of the particles, Lehninger and Cooper concluded that the inner membrane was comprised of repetitions

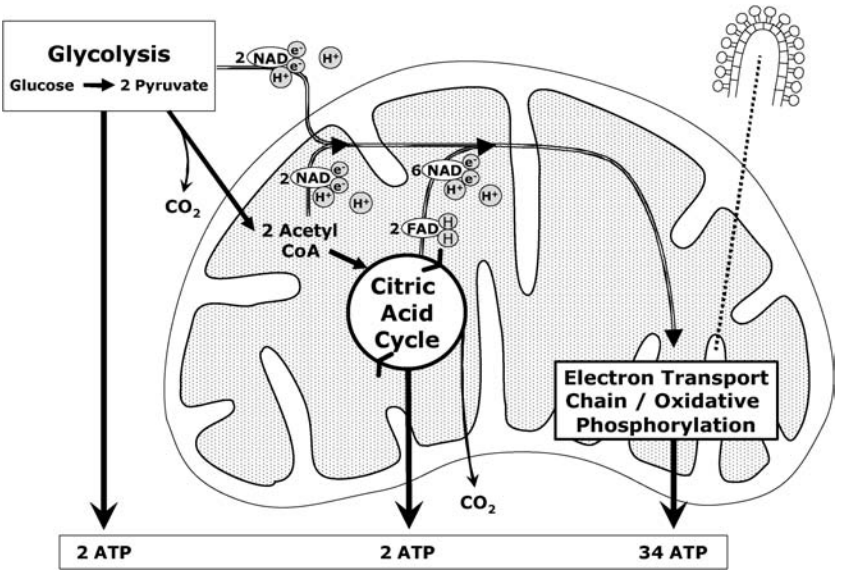

Figure 6.4. Localization of biochemical mechanisms of cellular respiration: (1) glycolysis in the cytosol; (2) citric acid cycle in the mitochondrial matrix; (3) electron transport and oxidative phosphorylation in the cristae.

of a basic respiratory assembly. Their objective was to determine the structure of one of these units, which they pursued through a series of experiments that focused selectively on the reaction between cytochrome *c* and oxygen (Cooper & Lehninger, 1956a; Cooper & Lehninger, 1956b; Devlin & Lehninger, 1956), on ATPase activity (Cooper & Lehninger, 1957a), and on ATP-P_i^{32} and ATP–ADP exchange reactions (Cooper & Lehninger, 1957b). The exchange reactions involved the regular exchange either of a free phosphate with one in ATP or the transfer of a phosphate from an ATP molecule to an ADP molecule. All of these reactions occurred in the digitonin prepared particles and were inhibited by decoupling agents. From these studies they concluded that phosphate and ADP enter into oxidative phosphorylation in separate, sequential steps. By investigating the exchange reactions in particular, Lehninger and his colleagues claimed to determine the order of the final events in phosphorylation. The alternatives were that the responsible enzyme (they used X, Y, and Z to represent the enzymes involved in each of the phosphorylation reactions) first reacted with the phosphate, creating a high-energy intermediate X∼P, etc., and then reacted with ADP to form ATP, or that they reacted first with ADP, creating X∼ADP, etc., and then with the phosphate. The evidence from the exchange reaction supported the former, not the latter, possibility (see Figure 6.5).

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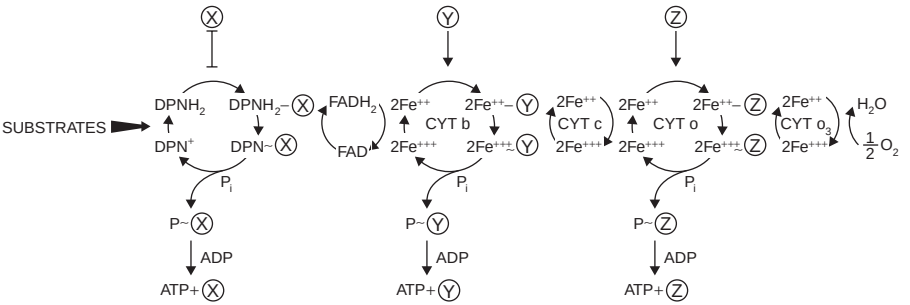

Figure 6.5. Lehninger's conception of the coupling of the electron transport chain with oxidative phosphorylation via high-energy intermediates. X, Y, and Z are three different enzymes, P_i is inorganic phosphate, ~ is a high-energy bond, and other substances are carriers undergoing reversible oxidation-reduction. Reproduced from A. L. Lehninger et al. (1958), Oxidative phosphorylation, *Science, 128*, 450–6, Figure 3, p. 455.

With this account of the final step, Lehninger and his colleagues considered two possible mechanisms (their term) for the generation of X~P. By one account it was the reduced carrier (e.g., the cytochrome receiving the pair of electrons) that entered into a high-energy bond with the enzyme, whereas by the other it was the oxidized carrier (the cytochrome surrendering the electrons) that formed the high-energy bond. Appealing again to evidence from exchange reactions, specifically, that the reactions occurred at a maximal rate when the carriers were kept in a fully oxidized state, but not in a reduced one, Lehninger and his colleagues concluded that the second proposal was correct. Although expressing caution as to how decisive the evidence was, they interpreted their results as supporting the mechanism shown in Figure 6.5 according to which, at each of the three phosphorylation sites, an enzyme bound itself to the reduced carrier. When the carrier was then oxidized, the bond between the carrier and the enzyme became a high-energy one. At the first site, for example, the oxidation resulted in DPN~X. Inorganic phosphate then replaced the carrier (DPN) in the bond, yielding P~X. Finally ADP replaced the enzyme (X), yielding ATP and X. Once this type of reaction sequence had occurred at all three sites, and the energy-depleted electrons had joined with hydrogen ions and oxygen to make water, one round of oxidative phosphorylation was achieved. Energy was now stored in high-energy phosphate bonds in several molecules of ATP and was available for a variety of purposes.

At this point, both Green and Lehninger had succeeded in segregating submitochondrial particles and had proposed accounts of the operations involved in the mechanism of oxidative phosphorylation. Efraim Racker then attempted to take the endeavor a step further by isolating a soluble enzyme that was

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responsible for the phosphorylation of ADP.[22] He employed a technique in which he fractionated mitochondria after breaking them with glass beads in a vacuum.[23] This produced a red-brown, gelatinous residue or particulate fraction that could oxidize some citric acid cycle intermediates but not accomplish phosphorylation unless the faintly turbid yellow supernatant was re-added. Then phosphorylation increased until reaching a P:O ratio of 0.5. He set about purifying the substance in the supernatant, which he labeled *coupling factor* F_1. At this point Racker followed up on a suggestion first advanced by Henry Lardy and Conrad Elvehjem (1945) that phosphorylation might be the inverse of the breakdown of ATP to ADP, attributed to the enzyme ATPase. He found that F_1 also exhibited ATPase activity, and after showing that both F_1 coupling and ATPase activity decayed at the same rate around 0°, identified them as the same protein (see Penefsky et al., 1960; Pullman et al., 1960; Racker, 1965, Chapter 13). Racker concluded, "F_1 catalyzes the transphosphorylation step from X∼P to ADP to form ATP at phosphorylation Sites 1 and 2" (1965, p. 169).

As the subscript number of the coupling factor suggests, Racker was also separating other factors – F_2, F_3, and F_4 – and investigating their role in phosphorylation or in other reactions such as ATP–P_i^{32} exchange. Of particular interest was factor F_o, which Racker first identified in the context of trying to account for the sensitivity of both oxidative phosphorylation and ATPase activity to oligomycin poisoning. Addition of F_o to F_1 not only provided oligomycin sensitivity, but when treated with salt solution, generated particles. I will discuss the significance of this discovery after introducing the discovery of one more morphological structure.

One More Piece of Structure and a Proposal as to Its Function

Yet another development in electron microscopy technique, the introduction of negative staining by Humberto Fernández-Morán (1962), revealed additional structure in the mitochondrion. Negative staining uses substances

[22] In his initial studies Racker collaborated with Gifford Pinchot to study oxidative phosphorylation in *Escherichia coli* in hopes of finding "a system which would withstand fractionation" (Pinchot, 1953, p. 65). They used sonic vibrations to prepare extracts and separated two components, a particulate fraction that catalyzed oxidation and a soluble fraction that was required for phosphorylation (Pinchot & Racker, 1951). Pinchot went on to study the reaction in *Alcaligenes faecalis*, where he distinguished two soluble fractions, one of which was heat labile and one of which was heat stable.

[23] This is a procedure Racker had previously used with tumor cells or bacteria. He offers an interesting characterization of his work as "instrumental research: When you run out of ideas, use a new instrument" (Racker, 1965, p. 164).

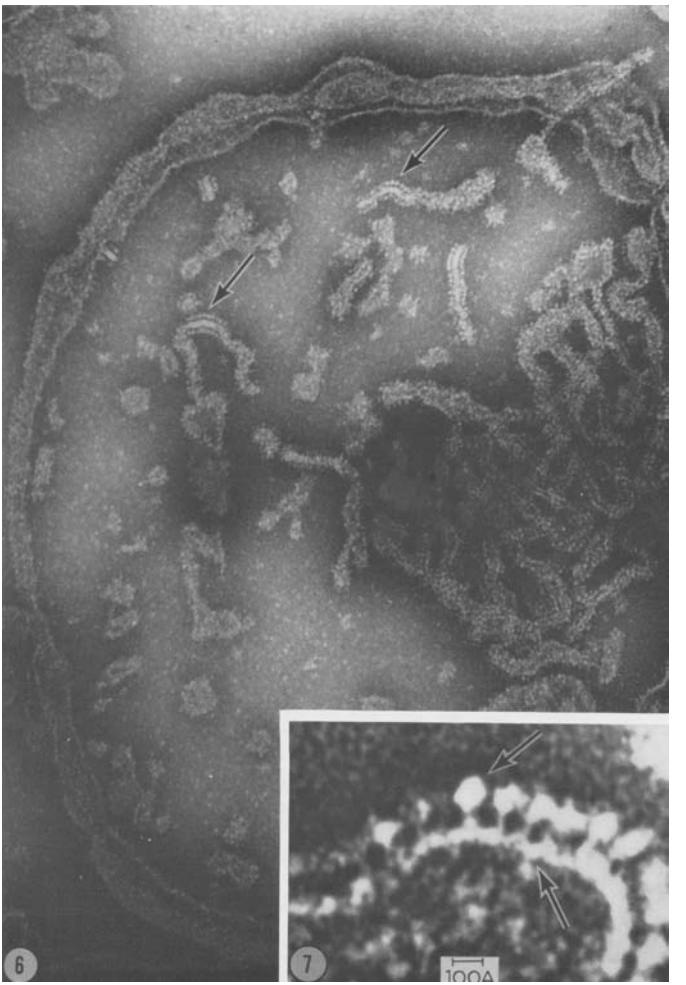

Figure 6.6. Fernández-Morán's negative-stained micrograph of cristae showing small spherical particles attached to the membrane. Reproduced from H. Fernández-Morán et al. (1964), A macromolecular repeating unit of mitochondrial structure and function: Correlated electron microscopic and biochemical studies of isolated mitochondria and submitochondrial particles of beef heart muscle, *Journal of Cell Biology*, *22*, 63–100, Figures 6 and 7, p. 73 by copyright permission of the Rockefeller University Press.

such as phosphotungstate or uranyl acetate that are electron dense but chemically inert. These substances do not react with membrane material, which then appears light against the dark background created by the electron dense material. Using this approach, Fernández-Morán discovered small particles (70–90 Å in size) located on stalks about 50 Å in length projecting from the

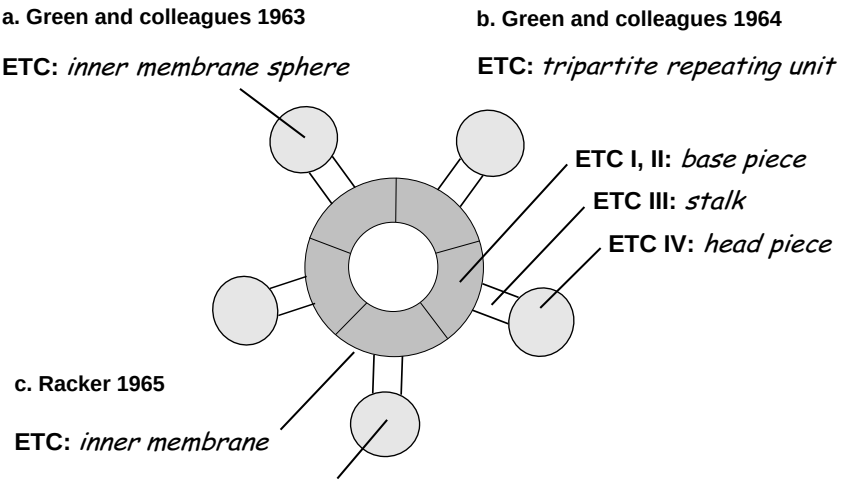

Figure 6.7. Three proposals as to the significance of the spherical particles on the mitochondrial inner membrane (cristae). (a) Green's 1963 proposal, in which each inner membrane sphere contained an entire electron transport chain (ETC). (b) Green's revised proposal of a tripartite repeating unit in which complexes I and II of the electron transport chain were contained in the base piece, complex III in the stalk, and complex IV in the head piece. (c) Racker's account localizing ATPase in the inner membrane spheres and the electron transport chain in the inner membrane.

cristae into the inner mitochondrial milieu. While small, these particles are numerous, with estimates of 10,000 to 100,000 per mitochondrion. When he applied the negative stains without prior fixation, mitochondria swelled and burst, extruding membranous material in the form of sheets, tubules, or ribbons that were studded with small spherical knobs about 90 Å in diameter. These spherical knobs can readily be seen (particularly in the inset) in Figure 6.6, which shows a micrograph Fernández-Morán published in 1964.

Green seized upon Fernández-Morán's discovery, naming the knobs *inner membrane spheres* and proposing that they constituted the complete system of enzymes for electron transport (Figure 6.7a). Lehninger (1964), however, calculated that the weight of the respiratory assembly was one to two orders of magnitude greater than that of these particles. As shown in Figure 6.7b, Green and his collaborators then proposed a distribution of the four different complexes of enzymes involved in electron transport over the base piece (Complexes 1 and II), stalk (Complex III), and head piece (Complex IV), respectively (Fernández-Morán et al., 1964). Although much of their analysis focused on the relative sizes of the stalk and head pieces and the minimum

sizes, based on molecular weight, of the enzyme complexes, ultimately they appeal to "biochemical considerations" to defend this localization: "Complexes I and II must interact with DPNH and succinate, respectively, both of which are localized in the interior of the crista, whereas complex IV must interact with molecular oxygen which would be more readily available in the solution outside the crista rather than in its interior" (p. 95). Green presented a popularized account of this proposal in a paper in *Scientific American* in 1964. There he also offered a speculative proposal (attributed to Robert Bock and Robert Criddle), according to which the transport of substrates between enzymes was accomplished "by means of swinging groups of atoms, mounted on the respective proteins by flexible arms, that transfer and accept the electrons" (Green, 1964). This mechanism, it should be noted, addressed the issue of electron transport, but not the accompanying phosphorylation.

Green's proposals were quickly discounted by other biochemists. I noted previously that Racker had found that F_o preparations generated particles when salt was added. Collaborating with Donald Parsons and Britton Chance, he examined these preparations with the negative staining technique and found that they were sac-shaped structures covered with "inner membrane spheres" like those found by Fernández-Morán. He then treated the preparations with trypsin, followed by urea, a treatment he had previously employed to remove ATPase activity from his preparations. Examining these preparations with negative staining, he found they had no inner membrane spheres. This established that, contrary to Green's proposal, the electron transport chain was not in the spheres. On the other hand, preparations of the F_1 factor showed spheres about 85 Å in diameter, and when such preparations were added to the trypsin-urea membrane preparation, spheres appeared on the membrane. The preparation still did not perform oxidative phosphorylation, but when F_2, F_3, and F_4 were added as well, phosphorylation was restored (Racker et al., 1965; see also Racker, 1968). He concluded that the spheres contained the ATPase and were the locus of ATP synthesis.

Racker was not fully satisfied with the demonstration, however, because the difference in number of spheres before and after adding F_1 was small enough that he had to rely on statistical analysis to establish it. His research assistant, Lawrence Horstman, tried passing mitochondrial fractions through a Sephadex column in order to remove the native spheres more effectively.[24]

[24] Racker (1976, p. 16) commented, "He tried these experiments without any encouragement from me because I did not think that the procedure could be carried out without damage to the particles. However, it worked, which brings us to the next lesson, Lesson 6: Progress is made by young scientists who carry out experiments old scientists said wouldn't work (F. Westheimer)."

This produced a far more definitive series of micrographs – one of submitochondrial particles with spheres, one with the spheres removed by Sephadex and urea, and a third, resembling the first, with the spheres reconstituted with F_1 (Racker & Horstman, 1967).

Racker's research localized the electron transport chain in the inner mitochondrial membrane and the ATPase in the spheres attached to the membrane (Figure 6.8c). This now presented a structural version of the problem biochemists had faced since the discovery of phosphorylation accompanying electron transport: How were the oxidation–reduction reactions of electron transport linked to ATP synthesis? The problem was now how to link activities localized in the inner membrane with activities in the attached spheres. For the most part, biochemists were still seeking chemical intermediates – the hypothetical compound C that, in Slater's scheme, formed an initial high-energy bond with the substrate and then transferred that bond to ADP or, in Lehninger's version (Figure 6.8), the postulated enzymes X, Y, Z that played that role at the three phosphorylation sites along the electron transport chain.

Radical Reconceptualization of Oxidative Metabolism

In 1961 Peter Mitchell, a maverick biochemist whose background had familiarized him with the enzyme-catalyzed translocation of chemical groups across membranes, advanced a revolutionary reconceptualization of oxidative phosphorylation. He suggested that the crucial intermediary was not chemical in nature but rather was a proton gradient across the inner mitochondrial membrane. The enzymes of the electron transport chain were so organized in the membrane that as the respiratory substrates were oxidized, protons (H^+) were discharged on one side of the membrane (the intermembrane space of the cristae) and OH^- ions were discharged on the other side (the mitochondrial matrix). Because the mitochondrial membrane is impermeable to H^+ and OH^- ions, a proton gradient bearing an electrical potential develops. When ATPase operates normally (that is, to hydrolyze ATP to ADP and inorganic phosphate), it also pumps ions across the membrane into the intermembrane space. But once a gradient has developed with significantly higher concentrations of protons in the intermembrane space than in the matrix, the ATPase can no longer can pump ions out of the matrix. This energy built up in the proton gradient provides the energy to drive the ATPase in reverse – synthesizing ATP from ADP and inorganic phosphate, rather than breaking it down (Mitchell, 1961; Mitchell, 1966). Because Mitchell's hypothesis, as shown schematically in Figure 6.8, made transport across a membrane

a central component of the mechanism, he called it the *chemiosmotic* hypothesis.

Mitchell's proposals were initially extremely controversial, giving rise to what are often referred to as the *ox phos wars* (Prebble, 2002). The opposition was partly empirical – for example, questioning the evidence for the claimed proton gradient – and partly theoretical. The idea of a proton gradient across a membrane was foreign to most biochemists, who were still oriented to the soluble systems model for explaining metabolic processes. Remarks of Efraim Racker reveal the combative nature of the debate: He referred to "hypothetical proton gradient and imaginary membrane potential" and compared Mitchell's claims to "pronouncements of a court jester or a prophet of doom" (Racker, 1975). Yet, at approximately the same time Racker wrote to the central figures investigating oxidative phosphorylation – Paul Boyer, Britton Chance, Lars Ernster, Tsoo E. King, Henry A. Lardy, and D. Rao Sanadi, in addition to Green, Lehninger, Mitchell, and Slater – proposing the preparation of a joint statement designed to reduce the acrimony over oxidative phosphorylation. Although negotiations over the joint review were tempestuous, several of the authors agreed on a joint introductory statement, followed by individual papers, that appeared in the *Annual Review of Biochemistry* for 1977 (Boyer et al., 1977).[25] By this time Racker had been convinced of the chemiosmotic hypothesis and his own contribution supported Mitchell's position. Although controversy continued, Mitchell was awarded the Nobel Prize in 1978, a testimony to the significance of his proposal in transforming thinking about the phosphorylation process.

With the incorporation of Mitchell's account of the linkage between electron transport and phosphorylation, the mechanism of oxidative phosphorylation was essentially resolved. Moreover, the account wove together in a fundamental way morphological structure with chemical operations. Palade's cristae not only were seen to contain the critical enzymes of electron transport in a spatially organized manner (the functional significance first attached to them), but also served to create the proton motive force that drove ATP synthesis. The stalks and spherical particles attached to the membrane housed F_0 and the ATPase and could respond to the proton gradient by synthesizing ATP. The combined contributions of studies of cell structure and biochemical function were melded into a comprehensive account of the mechanism that accounted for the phenomenon. Many details of the operation of the

[25] Racker (Interview, 1989, Ithaca, NY) expressed dissatisfaction with the final result since each author ended up arguing for his own position rather than engaging the others in the manner he had hoped.

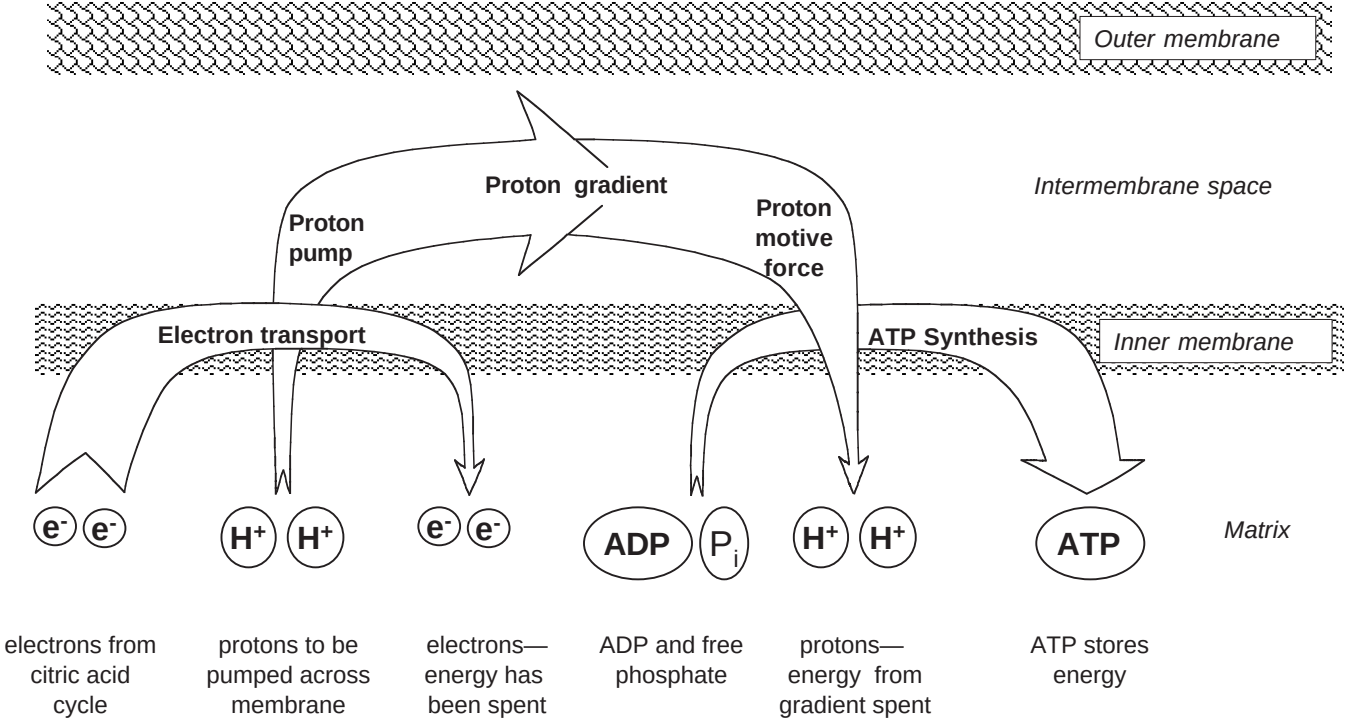

Figure 6.8. A schematic representation of Peter Mitchell's chemiosmotic hypothesis for oxidative phosphorylation. Arrows from left to right depict temporally ordered energy transfers, not spatial relations. As the width of the arrow representing each process increases (decreases), energy is gained (spent).

mechanism remained to be resolved, and this provided the focus of ongoing work in biochemistry. Although the locus of research on oxidative phosphorylation shifted after about 1970 to biochemistry, the research in the prior decades was an outstanding exemplar of research in the new field of cell biology. By revealing structure and organization at a level between older biochemistry and traditional cytology, de Duve's terra incognita had become an explored territory.

2. MICROSOMES, THE ENDOPLASMIC RETICULUM, AND RIBOSOMES

From Lace-like Reticulum to Endoplasmic Reticulum

As I discussed in the previous chapter, Claude first identified microsomes through cell fractionation while Porter, examining his electron micrographs, described a lace-like reticulum with granules and identified Claude's microsomes with what appeared as granules in it. Although Claude remained agnostic, many investigators proposed that microsomes were involved in protein synthesis. At the same time as research on the mitochondrion was revealing the mechanism of oxidative phosphorylation, researchers were making rapid advances in their understanding of these additional cytoplasmic structures, an endeavor that culminated in an account of how the structures figured in protein synthesis. Porter played a key role in initiating these developments. In papers published in 1952 and 1953 he, together with Frances Kallman, a postdoctoral fellow of the National Cancer Institute, captured images of this lace-like reticulum by increasing the time of fixation in osmium vapors. Porter proposed that the osmium vapor digested the "diffuse and frequently fibrous components of the ground substance" leaving "what may be thought of as a membrane skeleton of the cell" (Porter & Kallman, 1952, p. 883). With this technique, Porter and Kallman provided a further description of Porter's lace-like reticulum and gave it a new name:

> A third component uniformly present in these images is made up of vesicular or canalicular elements which sometimes constitute a complex reticulum. This material is part of the innermost cytoplasm of the cell, the endoplasm. It is referred to as the endoplasmic reticulum from its location and form. It appears to be a finely divided vacuolar system. It varies enormously in different cells in the size of its division and, though its function is not known, this variation reflects in part the physiological state of the cell at the time of fixation. Phase contrast microscopy provides evidence of its presence in the living cell. (p. 883)

Porter and Kallman then turned their attention to the particle that Porter and Thompson had observed in tumor cells and which by then had been reported by two other electron microscope laboratories (Cannan & Berger, 1951; Oberling et al., 1950). Porter and Thompson had found these particles to be limited to tumor cells and now Porter and Kallman suggested a reason: "We were . . . comparing rapidly proliferating tumor cells with 'resting' normal cells" (p. 887). They reported finding such particles in actively growing cells derived from young rat heart embryonic tissue. Because they appeared when cells, normal or tumor, were rapidly growing, they proposed "to associate these granules with growth processes in the cell, *i.e.*, in the production of new protoplasm, and they have been tentatively referred to as growth granules" (p. 887). Relying on results from absorption studies with ultraviolet microscopy (Ludford, Smiles, & Welch, 1948), which indicated a nucleotide composition of the particles, they concluded that the particles "may have a high content of ribo-nucleotides, which might be expected if they are accepted as multiplying components of the cytoplasm" (pp. 888–9). They went on to associate the particles with the microsomal fraction from fractionation studies. In what they admitted to be speculation, they continued,

> it is attractive to think of them as centers of synthesis of all cytoplasmic components. There is some preliminary evidence from the micrographs that mitochondria may begin their development in this form, but elements of the endoplasmic reticulum, the lipid granules, and inclusions, the distinctive features of differentiated cells, may be similarly derived. If such is the case, we are led to postulate that there are several subspecies among this class of cytoplasmic particles and that the complement of these in any cell would determine the type of differentiation to some extent. (p. 890)

Through the 1940s Porter's description of a lace-like reticulum and his suggestions of its function failed to attract much response from other investigators. This was largely because the structure appeared only in micrographs of whole, cultured cells, which he nearly alone was making. This changed with the improvement in techniques for making micrographs of ordinary thin-sliced cells. Albert Dalton (1951a; Dalton et al., 1950) published electron micrographs which showed a number of filamentous units in the cytoplasm, which tended to be grouped in particular areas and which were reduced in number when the animal fasted.[26] Soon thereafter, Wilhelm Bernhard and

[26] "Differentiation of filament-like stands are present in the cytoplasm of the proximal tubule cells but they have been found only in the basal parts of the cells and are somewhat thinner (approximately 0.05μ) than cell membranes. They are also identifiable by the fact that they terminate in the cytoplasm without returning to one of the tubule surfaces. . . . These structures

Charles Oberling (Bernhard, Gautier, & Oberling, 1951; Bernhard et al., 1952) also reported finding ergastoplasmic filamentous or lamellar structures (see Haguenau, 1958, for a review).

Noting Dalton's report of filaments in thin sections, Palade and Porter, when they started making micrographs of thin sections in 1950–1, investigated the relation between these and Porter's endoplasmic reticulum. They reported that with buffered osmium the "'filaments' were in fact fine tubules, or strands of vesicles identical in size and form to those constituting the endoplasmic reticulum of cultured cells" (*Annual Report*, 1950–1, p. 147). In his 1953 paper with Blum describing their new microtome, Porter presented a micrograph of a thin section in which he identified

> numerous 'elongated elements' and many 'granules'. These vary from 50 mμ to 150 mμ in diameter and much more in length. When they are examined carefully they are found to have a relatively 'transparent' center bounded by a single dense line apparently representing a membrane. These elements of the cytoplasm are *easily recognized* as the equivalent of the endoplasmic reticulum indicated previously in cultured cells (Porter & Thompson, 1948) and identified with the basophilic component of the cytoplasm (Palade & Porter, 1952; Porter, 1953). (Porter & Blum, 1953, p. 699, emphasis added)

Although Porter and Blum claimed the identification is easy, one thing that was clearly lost in the micrographs made of thin slices was the three-dimensional perspective offered with whole tissue-cultured cells. Without commenting on how this had already led to different interpretations of the endoplasmic reticulum, Porter and Blum stated, "In any further study of this endoplasmic reticulum it is important to determine its three-dimensional form in cells fixed *in situ* in their respective tissues. Obviously for this and similar problems serial sections of the cells are essential" (p. 700). They then presented a series of four serial sections and commented,

> it is observed that the system referred to as the endoplasmic reticulum is a complex of interconnected strands and sinusoids, the latter tending to be flattened in one dimension but otherwise very polymorphic. At certain sites in this parotid

are, as a rule, oriented in a position perpendicular to the basement membrane. An attempt has been made to determine whether they are filaments or actual lamellae" (Dalton, 1951a, p. 1167). From the fact that they stay in focus while the focus of the electron microscope is changed, Dalton concluded they are lamellar in form. In a review in 1953, Dalton argued, "In our preparations the components of this material were usually found as long, sometimes branching, parallel or concentric strands and never in cross section as solid or hollow spheres of the same diameter as the strands. This naturally suggested that they are lamellae rather than filaments or tubules" (p. 411).

> cell these flattened portions of the system are organized in parallel arrays but in other regions of the cytoplasm the system is represented by finer canalicular or vesicular elements. If the serial micrographs are examined closely instances may be found where discrete elements appearing as cross sections of canaliculi come together progressively into single elements representing either longitudinal sections through canaliculi or, more likely, marginal sections through sinusoids. Also in the same series, sequences are apparent defining arborizations of unit structures. In still other regions of the cytoplasm the only elements evident appear as tiny vesicles or cross sections of canaliculi. Since certain of these can be traced from section to section they are evidently segments of canaliculi. (p. 700)

Porter's claim that the structures apparent in whole tissue-cultured cells were the same as those that could be reconstructed across a series of thin sections of cells *in situ* soon turned contentious. Critics objected that what appeared in tissue-cultured cells was an artifact of the process of growing cells in such an abnormal environment. To address this objection, Palade and Porter (1954) adopted the strategy of preparing thin sections of tissue-cultured cells to see how these would look and compared a series of them to the usual whole mounts of such cells. By demonstrating correspondences, they hoped to legitimate the use of micrographs of whole tissue-cultured cells.

Before presenting their results, they advanced a theoretical claim as to how the sections of tissue-cultured cells should look: "Under such circumstances the endoplasmic reticulum . . . cannot be expected to appear in sections as a network. Occasionally, it could be included in the thickness of a given section, but in the vast majority of sections only profiles of 'vesicles and strands' will be encountered and these will appear as independent structures because their original connections have been severed by the microtome" (p. 664). Palade and Porter presented micrographs of a sectioned chicken monocyte (white blood cell) as well as a whole-mount and sectioned macrophage grown from monocytes in tissue culture to establish the correspondences in appearance between whole mounts and thin slices. They went on to compare whole mounts of cultured cells and thin sections of various mammalian cells fixed *in situ* to make their case (see Figure 6.9).

In a second paper in the series, of which he was the sole author, Palade described the appearance of the endoplasmic reticulum in cells in several tissue types from rats and chickens – epithelial, nervous, mesenchymal, and muscular. One difference Palade observed between cells from living organisms and those from tissue culture is that in cells from living organisms the reticulum runs from the nuclear membrane to the cell membrane. Thus, it occurs in both endoplasm and exoplasm. Noting this and the fact that "the

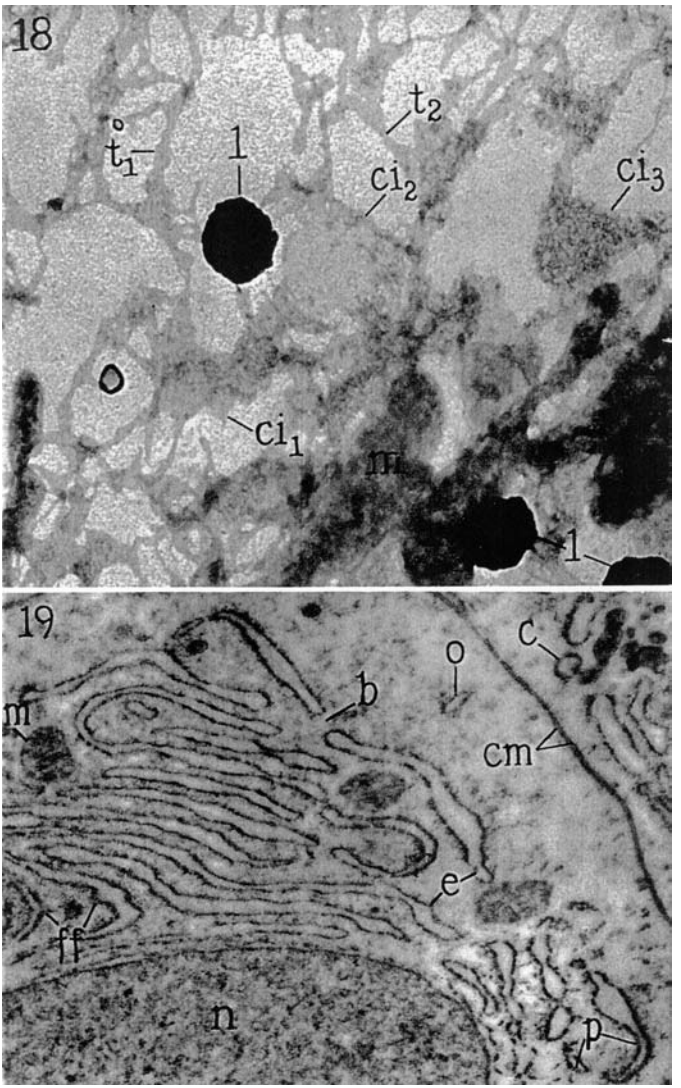

Figure 6.9. A pair of micrographs in which Palade and Porter compared the appearance of the endoplasmic reticulum in whole tissue-cultured cells (top) and in thin sections (bottom), both from glandular epithelia of the parotid of a newborn rat. Reproduced from Palade, G. E., & Porter, K. R. (1954). Studies on endoplasmic reticulum: I. Its identification in cells *in situ*. *Journal of Experimental Medicine*, 1954, *100*, 641–56, Plate 62 by copyright permission of the Rockefeller University Press.

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word 'reticulum' was stretched a little to accommodate preferentially oriented dispositions of the system," Palade asserted, "the name 'endoplasmic reticulum' has a number of admitted shortcomings. We retain it because we do not have a better one" (1956b, p. 92).[27]

For Palade and Porter the micrographs of whole cells provided an anchoring point to which they appealed in interpreting thin sections, and Palade noted the problems others had in interpreting thin sections without this reference point:

> The usual thickness of such sections, *i.e.* 20 to 40 mμ, being much smaller than the mesh size of the reticulum, and even smaller than the diameter of the vesicles and tubules that form its trabeculae, it follows that in sections only slices of these trabeculae or "profiles" can be found. Fragments of meshes, or more or less complete meshes are only occasionally encountered and the continuity of the system throughout the cytoplasm is never apparent; it has been lost by microtomy and can be regained only by the difficult and tedious operation of tridimensional reconstruction. In this respect, one may comment that the use of spread cells as initial specimens for the study of the endoplasmic reticulum was a fortunate coincidence. The main feature of the system, *i.e.* its disposition in a continuous reticulum that permeates the entire cytoplasm, would have been extremely difficult if not impossible to grasp from an exclusive study of sections. It is then easy to understand why, at the beginning at least, the shift from spread to sectioned specimens caused so much confusion about the endoplasmic reticulum. In the few years that have elapsed, a number of conflicting descriptions and interpretations have been advanced and a corresponding number of names proposed for the structures belonging to the system under consideration. A vacuum in terminology, be it only apparent, seems effectively to lead many a biologist into philological temptation. (1956b, pp. 86–7)

In some cases, however, Palade and Porter found clues in the thin sections that allowed them to reinterpret micrographs of whole cells:

> The examination of serial sections and of sections of various incidences indicates that the elements in question are relatively large, flattened vesicles of irregular outline, but of shallow and relatively constant depth for which the

[27] The name did survive even though many critics tried to displace it. In particular, French authors such as Françoise Haguenau (1958) sought to maintain Garnier's term *ergastoplasm*. Novikoff (1956b, p. 971) commented, "Agreement on terminology, sometimes made more difficult by considerations of national pride and human personality, appears to be close at hand, as we rapidly learn more about the membrane systems of cells. Porter's work (1955–6) suggests that all cells possess a similar basic component – a vacuolar system of great complexity – and that in different cells this system shows varying degrees of continuity and specialization. Thus, according to this view, 'ergastoplasm' is essentially a specialized type of 'endoplasmic reticulum' characterized by the presence of basophilic granules on its surface."

> name of *cisternae* has been recently proposed (Palade & Porter, 1954). Because such elements were not originally described in cultured cells examined *in toto* (Porter, 1953), spread specimens were reexamined (Palade & Porter, 1954) and in many cases the endoplasmic reticulum was found to consist mainly of large, flat cisternae of irregular outline with only a few tubular and vesicular elements present. (Palade, 1956b, p. 89)

Just as he did in the case of mitochondria, Palade observed in his improved micrographs a new structure in the endoplasmic reticulum – he found that frequently the outer surface of the membranes of the endoplasmic reticulum were coated with fine granular material that at higher resolutions appeared as discrete structures ranging from 10 to 30 mμ in size.[28] Porter (1954), after arguing that the endoplasmic reticulum was the source of the basal staining and hence the basophilic component of the cytoplasm, concluded that the basophilia was due to these particles on the grounds that the basal staining was most prominent in tissue in which granules were attached to the endoplasmic reticulum. He further related the granules to a fraction with particles smaller than the microsomes isolated by Barnum and Huseby (1948) and to a fraction of particles of the same size as those on the endoplasmic reticulum that Petermann, Mizen, and Hamilton (1953) demonstrated to contain large amounts of RNA. Porter concluded by identifying similar particles in the nucleolus.

Dissenting Voices

As in the case of the mitochondrion, Sjöstrand criticized Porter and Palade's studies of the endoplasmic reticulum. Emphasizing the fact that in thin slices what appeared were pairs of membranes, Sjöstrand and Rhodin claimed that the ground cytoplasm was divided "into open compartments through well-defined intracellular double membranes" (1953, p. 428). The following year, in a paper with Hanzon, Sjöstrand expanded on his characterization of the intracellular cytoplasmic membranes, now identifying "'small opaque particles' attached to one side." (These are quite apparent in the micrographs they published – see Figure 6.10.) He further described,

> The smooth surfaces of the intracellular cytoplasmic membranes face each other giving the impression of the membranes being arranged in pairs. They always

[28] Porter's initial response to these particles on parts of the endoplasmic reticulum was that they were likely artifacts due to damage caused by the electron beam. With a denigrating intent, he initially dubbed them *Palade particles*. (Interview with George Pappas, 23 October 1995, University of Illinois Chicago.)

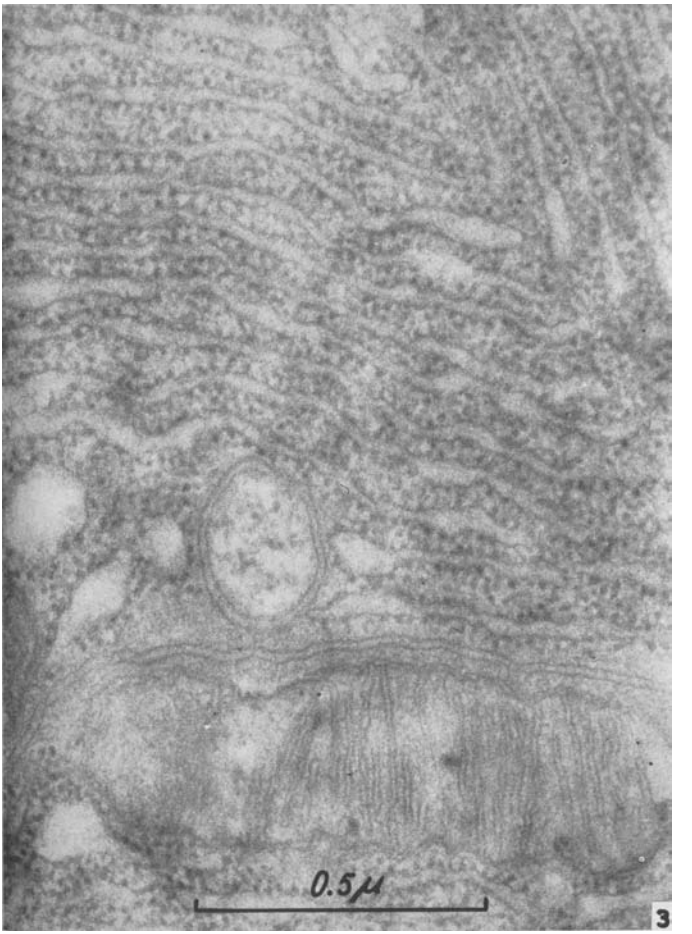

Figure 6.10. Sjöstrand and Hanzon's micrograph of paired intracellular membranes with small opaque particles attached on one side of each membrane. At the bottom a mitochondrion is shown. Reproduced from F. S. Sjöstrand and V. Hanzon (1954), Membrane structure of cytoplasm and mitochondria in exocrine cells of mouse pancreas as revealed by high resolution electron microscopy, *Experimental Cell Research, 7*, 393–414, Figure 3, p. 397, with permission of Elsevier.

face the mitochondria, the zymogen granules and the cell membrane with their rough side but the cytoplasmic membranes adjacent to the nuclear membrane always have the smooth surface directed towards the nuclear membrane. Therefore at least one of the membranes appears to be a single membrane. (Sjöstrand & Hanzon, 1954, pp. 403–4)

Sjöstrand further noted that:

> When the cytoplasmic membranes are cut obliquely or are oriented parallel to the plane of the section the basic membrane does not show up distinctly or is not visible at all due to its low electron scattering capacity. Then, only the opaque particles are observed and the cytoplasm appears as consisting only of this component. (p. 405)

From the fact that the particles do not appear in freeze-dried preparations but appear very opaque in osmium tetroxide preparations, he proposed that they react very strongly with osmium. Late in the paper Sjöstrand seemed to jump to a conclusion about the function of these particles. From the fact that they "represent the dominating structure of the cytoplasm of the exocrine pancreas cells," he inferred that "they are structures of importance for the enzyme synthesis in these cells" (412). This correct inference is surprising both in light of his failure to offer justifications for it and the fact that in the case of the mitochondrion Sjöstrand resisted any speculation about function.

Sjöstrand also linked these paired membranes to structures identified by other electron microscopists – the "interdigitating cell membranes" of Pease and Baker (1950) and the intracellular filaments or lamellae of Dalton et al. Sjöstrand commented that "the micrographs of these authors are, however, not of the quality to allow a detailed description and a correct interpretation of these structures" (p. 448). In particular, he rejected the interpretation that these structures are filaments. In his paper with Rhodin, he claimed:

> It is quite obvious that we are dealing with membranes and not filaments from the fact that they may be followed without interruption through the whole basal cell zone, the chance to hit a filament so exactly along its entire length being negligible. In addition, there have never been any indications of cross-cut filaments. (p. 448)

In the paper with Hanzon he advanced a further argument – the number of membranes was always odd, due to the membrane closest to the nucleus not having a partner. This would not be the case if the structures were filaments.

At a symposium at the Eighth Congress of Cell Biology in 1954, Sjöstrand related his views to those of Porter and Palade:

> It is of course very threatening [tempting?] to generalize regarding these different structures and to consider for instance the opaque particles as a new component of the cytoplasm of general occurrance (sic) (Palade, 1953) with a common chemical and functional significance. For the moment we may not generalize further than accepting that in the cytoplasm there exist granules

> of different sizes and topographical relationships and with one property in common, that they react intensely with osmium tetroxide. But as this means a rather unspecific reaction these granules might chemically be of rather different types.
>
> As to the membranes observed in the cytoplasm these membranes certainly are morphologically very different. It might be possible to propose a definition that would characterize some of these membrane structures and would collect them as morphologically similar. To such morphologically well defined membranes a name could be given. The naming would, however, not increase our understanding but would represent a piece of systematic work. The term "endoplasmic reticulum" (Porter, 1953) is used in a too vague way, it almost indicates anything in the ground substance of the cytoplasm. Without guarantee for homology such diffuse classification certainly does not help very much in systematizing the structural components of protoplasm. (Sjöstrand, 1955b, pp. 226-7)

Securing the Connection to Protein Synthesis

While Porter and Palade were trying to determine the structural character of the endoplasmic reticulum, biochemists in a different line of research were following up on Claude's identification of microsomes in his fractionation studies. Shortly after he discovered microsomes, Claude had noted their high RNA content and, as discussed in Chapter 5, Brachet and Caspersson linked RNA to protein synthesis. Several biochemists attempted a direct assault on the problem of protein synthesis. They saw the formation of peptide bonds between amino acids to form polypeptide chains (Figure 6.11) as the fundamental step in protein synthesis. Their strategy was to trace the uptake of radioactively labeled amino acids into protein and identify the fraction in which it appeared. Harry Borsook of the California Institute of Technology (Borsook et al., 1950) and Tore J. M. Hultin of the Wenner-Gren Institute in Stockholm (Hultin, 1950) were early pioneers, independently showing in 1950 that when the cells of a labeled tissue were broken and fractionated, the highest concentration of labeled amino acids showed up in the microsome fraction.

Paul Zamecnik, at the Huntington Laboratory of Massachusetts General Hospital, played a particularly important role in these biochemical studies. When he initiated his work, two ideas on protein synthesis dominated the landscape. Max Bergmann at the Rockefeller Institute, with whom Zamecnik worked briefly before taking up his position at the Huntington Laboratory, proposed that cathepsin enzymes, which catalyzed proteolytic reactions, might synthesize peptide bonds. Fritz Lipmann, his colleague at Huntington, proposed that a phosphorylated intermediate might play a central role in causing

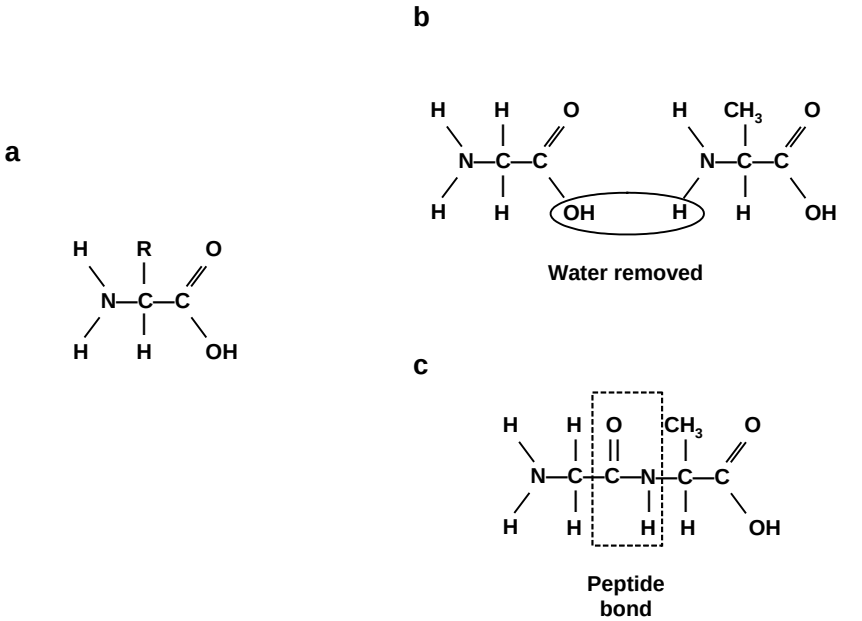

Figure 6.11. Basic steps in creation of peptide bonds in proteins. (a) Generic structure of an amino acid, where R represents the portion in which different amino acids vary. (b) Glycine (left) and alanine (right) bonding together with the removal of a water molecule. (c) The resulting peptide bond formed between glycine and alanine.

amino acids to bond to one another.[29] (Lipmann, as discussed earlier in this chapter, characterized such phosphate bonds as energy-rich bonds and theorized about how they were formed in oxidative phosphorylation.)

Robert Loftfield, who joined Zamecnik's group in 1948, had developed a procedure for labeling the amino acids alanine and glycine with C^{14}. In their first studies using these labeled amino acids, Zamecnik and his collaborators (Zamecnik et al., 1948) demonstrated uptake of alanine and glycine into tissue slices from normal and malignant rats. Working with tissue slice preparations presented serious limitations, and a number of research laboratories set out to develop a cell-free system in which to study the process.[30]

[29] Zamecnik commented, "As a student of Bergmann, I felt a loyalty to the catheptic enzymes, but as a neighbor of Lipmann, I developed a feeling that his concept of a phosphorylated intermediate might be correct, and the conviction that in any case C^{14}-labeled amino acids were a tool which might resolve this dilemma" (1958–9, p. 258).

[30] In Zamecnik's group, Elizabeth Keller initially led this effort. She injected rats with low doses of labeled amino acids and later sacrificed the animals, formed a homogenate from their liver cells, and centrifuged it. In rats sacrificed within twenty minutes she found most of the labeled amino acids in the microsomal fraction, but not after longer delays (Keller, 1951).

Philip Siekevitz, who joined the Zamecnik group in 1949 as an NIH Fellow, adapted the techniques developed by Hogeboom et al. at Rockefeller to create a fraction that contained both mitochondria and microsomes in which he could study incorporation of labeled amino acids. When he added citric acid cycle intermediates, incorporation increased. If respiration were suppressed, he found adding ATP could foster incorporation (Siekevitz & Zamecnik, 1951; Siekevitz, 1952). These were important clues to the energetics of protein synthesis and offered support for Lipmann's conception.

Yet further enhancements in the fractionation procedure, especially use of the Potter-Elvehjem homogenizer, enabled Nancy Bucher (1953) in Zamecnik's laboratory to demonstrate incorporation of labeled acetate into cholesterol and subsequently into proteins. Using this preparation Zamecnik and Elizabeth Keller showed that it was the microsomal and supernatant fractions that were required for protein synthesis, and that they had to be supplemented by ATP and guanosinetriphosphate (Keller & Zamecnik, 1956). Then, in collaboration with Mahlon Hoagland, Zamecnik and Keller revealed that the ATP interacted with the amino acid, forming an amino acyl ~ AMP compound, in the soluble protein fraction (Hoagland, Keller, & Zamecnik, 1956; Hoagland, 1955). They construed this as *activating* the amino acid, thus enabling it to form a peptide bond with another amino acid. With this research, the Zamecnik group (a) secured the claim that Claude's microsome fraction was the locus of protein synthesis, and (b) demonstrated the dependency of these activities on energy made available by the mitochondrial system.

Integrating Morphology and Biochemistry

In 1952 Siekevitz left Zamecnik's group to go to Wisconsin to work on oxidative phosphorylation with Van Potter. Three years later, George Palade recruited him to the Rockefeller Institute. After Hogeboom and Schneider had left, the Rockefeller group had not had a researcher primarily trained in biochemistry and had focused their efforts on electron microscopy. Those efforts had paid off handsomely, but it was now necessary to figure out the operations performed by the differentiated components, especially the laminar membranes of the endoplasmic reticulum and the small particles that dotted their surfaces in many places. With Siekevitz providing biochemical expertise, Palade immediately set out to conduct an "integrated morphological and biochemical study" of microsomes, initially from liver and subsequently from pancreatic acinar cells. They examined a portion of the specimen at each step in the fractionation process under the electron microscope. In the homogenate they identified "'hollow' profiles comparable in size, shape, and number to

the profiles of the endoplasmic reticulum in intact cells" (Palade & Siekevitz, 1955, p. 178). Noting that some of the microsomes contained small dense particles attached to the outside of their limiting membrane, they argued for identifying microsomes with the rough endoplasmic reticulum. They further commented that preparing the homogenate appeared to cause "an extensive fragmentation of the networks into independent vesicles, tubules, and cisternae, which subsequently can be centrifuged down, together with other cell components, into the homogenate pellets" (p. 179). There was no evidence of a tearing of the membrane, leading them to infer that the fragments "'heal' easily" or, more likely, are formed by a "'spontaneous,' generalized pinching-off process" (p. 192). Palade and Siekevitz concluded that Claude was correct in treating the microsomes as preformed components of the cytoplasm, but wrong to think of them originally as independent particles – rather, they are parts of a "continuous, cell wide system; i.e., the endoplasmic reticulum" (p. 190). This identification provided a bridge between the biochemical investigations of microsomes and the electron microscopy studies of the endoplasmic reticulum and its particles.

Once the homogenate had been centrifuged into microsomal pellets, Palade and Siekevitz found "membrane-bound profiles of approximately the same size and shape as the profiles found in homogenate pellets and considered to be derived, by extensive fragmentation, from the endoplasmic reticula" (p. 179). They observed that particles were still attached to many of the membranes, although they were "slightly less numerous" than in the homogenates, a factor that led them to try to fractionate further the microsomal supernatant in order to isolate the particles. They failed with liver preparations but were more successful in pancreas preparations, where the resulting particles exhibited high RNA and protein content but very low phospholipid content. They had greater success treating the microsomal fraction chemically – deoxycholate treatment eliminated the membrane, leaving the particles, while versene treatment and ribonuclease treatment eliminated the particles, leaving the membrane. From this, they conclude that the RNA is found in the particles and that the protein, phospholipids, and other components associated with microsomes are found in the membrane.

A further consideration in identifying RNA with the particles was that in many cell types the particles often appeared independently of the endoplasmic membrane. Especially in developing cells in intestinal epithelium, whose cytoplasm stains broadly with basic dyes, Palade reported finding "numerous free particles evenly and randomly distributed throughout the cytoplasmic matrix." In contrast, "the endoplasmic reticulum is represented by only a few vesicles and tubules, many of them free of attached particles, and relatively

large expanses of the cytoplasm contain no elements of the network" (1958a, p. 289).[31] If the particles could exist independently of the endoplasmic membrane, what was the significance of their association with the membrane in many adult cells? Palade speculated,

> it may be assumed that the reticulum provides appropriate surface for the arrangement of the small granules, but thus far we do not know whether the patterns described have any significant role in protein synthesis. Available information suggests, however, other possible reasons. In certain cells, for instance, the cavities of the endoplasmic reticulum appear to be in continuity, at least intermittently, with the extracellular medium through invaginations of the cell membrane. In such cases the supposedly fluid phase that occupies the cavities of the network may contain a variable amount of the extracellular fluid. It is possible that many raw materials, coming from outside into the cytoplasm, reach the particles through the labyrinth of the endoplasmic reticulum. In other cells the cavities of the endoplasmic reticulum appear to be used for the storage of a cell product. In plasma cells, thyroid epithelia, and fibroblasts they are frequently found distended and filled with an apparently amorphous mass of appreciable density. There are, in other words, numerous appearances suggesting that the cavities of the reticulum serve as feeding channels and storage space for the activity of the particles. Their association may have the same significance as the location of our plants along convenient ways of communication. (p. 301)

From the perspective of developing a model of a mechanism, Palade has here offered a decomposition into distinct operations: supplying materials, synthesizing proteins, and transporting the products. Palade (1958b) further developed this perspective, drawing insights from the nineteenth-century research of Rudolf Peter Heinrich Heidenhain (1875), who had found that in exocrine cells of the pancreas, granules disappear after food intake and are replaced with new granules a few hours later. Heidenhain concluded that the granules were comprised of the precursors of the digestive enzymes of pancreatic juice and that they provided temporary storage for these proteolytic enzymes. He named them zymogen granules. Following up on Heidenhain's research, Siekevitz and Palade compared the appearance of the endoplasmic reticulum in cells from guinea pigs starved for forty-eight hours with cells from guinea pigs fed an hour before. In the recently fed animals, "the cavities

[31] A note indicates that the text of this paper was prepared for a talk in February 1955, and was largely unaltered until publication. Palade commented on the rather unusual patterns of the particles on the endoplasmic reticulum membrane: "Sometimes, while looking at these intriguing patterns, I believe that I feel very much like the French explorers who, during Napoleon's expedition to Egypt, found themselves face to face with the hieroglyphs. Like some of them, I am recording the patterns, and I am waiting hopefully for a biochemical Champollion to decipher their meaning."

of the system are distended, the preferred orientation is lost, and relatively large, dense granules are found within the cavities of the distended cisternae" (p. 71). These granules were similar to, but smaller than, the zymogen granules. Fractionating cells from both starved and fed animals, they found that in starved animals there was no appreciable proteolytic enzyme activity in microsomes, but that in recently fed animals it approximated that of the zymogen particles. By further fractionating the microsomes after treatment with doxycholate, they showed a higher concentration of the proteolytic and ribonuclease activity in both the particles and in the whole microsome for recently fed animals than for starved animals. Palade concluded,

> To my knowledge, this is the first instance in which a product of the endoplasmic reticulum has been demonstrated in the form of well defined granules within the cavities of the system, and has been identified biochemically. (p. 73)

Palade was cautious about concluding that these enzymes were new products of protein synthesis, but did cite evidence using labeled leucine-ɪ-C^{14} showing its earliest incorporation occurred in the particles still attached to the membrane. A little later the label was found in the intracisternal granules and only later in the zymogen granules. This pattern, Palade contended, is "compatible" with the hypothesis that the microsomal particles synthesized the new protein.

Naming the Ribosome

In February 1958, the new Biophysical Society held its first symposium at MIT, with a focus on microsomal particles and protein synthesis. One of the most important results of the meeting was the adoption of a new name, *ribosome*, for the particles. The editor, Richard Roberts, related:

> During the course of the symposium a semantic difficulty became apparent. To some of the participants, microsomes meant the ribonucleoprotein particles of the microsome fraction contaminated by other protein and lipid material; to others, the microsomes consisted of proteins and lipids contaminated by particles. The phrase "microsomal particles" does not seem adequate, and "ribonucleoprotein particles of the microsome fraction" is too awkward. During the meeting the word "ribosome" was suggested; this seems a very satisfactory name, and it has a pleasant sound. The present confusion would be eliminated if "ribosome" were adopted to designate ribonucleoprotein particles of the size range 20 to 100 Å. (1958, p. viii)[32]

[32] Rheinberger (1997, p. 190, n. 12) claims that the suggestion stemmed ultimately from Howard Dintzis.

This symposium also marks a threshold in the research on the endoplasmic reticulum and ribosomes. Roberts noted further in his introduction that the case for protein synthesis by the ribosomes was still inconclusive. Several of the reasons he listed had to do with technical problems in demonstrating the connection, but one focused on the absence of a conception of the responsible mechanism: "No mechanism has been suggested which shows how the structure of the particle is compatible with its function as the template for synthesis of long chains" (p. vii). The mechanism that Palade had described treated the ribosome as a unit with an operation which interacted with the operations of the endoplasmic reticulum. It had not explained the operation of the ribosome itself.

Discovering the mechanism by which the ribosome synthesized proteins required moving to a yet lower level of organization at which research could focus on the chemical structures that comprised the ribosome or interacted with it. As was the case with the mitochondrion, research at this level was chiefly the province of biochemists and practitioners of two other new disciplines, biophysics and molecular biology, not researchers affiliating with cell biology. Since the research contributing to a basic sketch of the mechanism of protein synthesis in the ribosome is illustrative of one strategy for developing an account of a mechanism, I will briefly analyze it before returning to research in cell biology focusing on the transport of the newly created proteins.[33]

Going to a Lower Level: Decomposing the RNA Machinery

The biochemical research so far focused only on the process of making peptide bonds, but synthesizing proteins required linking amino acids in appropriate orders. Zamecnik (1958) proposed that protein synthesis involved "some biological equivalent of a printing press" that would specify the order and that the "press or template . . . is very likely RNA" (p. 120). He then advanced a scheme, initially proposed by Victor Konigsberger and Theo Overbeek, according to which amino acids are first activated by binding with an enzyme and a molecule of ATP, resulting in the transfer of the phosphate bond to an amino acid. He proposed that the amino acids bonded sequentially on the RNA template, and by what he characterized as a "zipper reaction" bonds were established between adjacent amino acids. In the final step in his

[33] For a discussion of the discovery of the mechanism of protein synthesis that emphasizes the interaction of molecular biology with biochemistry, see Darden and Craver (2002).

schema for the mechanism, the amino acid chain then separates, binds with other chains, and folds into the protein molecule.

Zamecnik and his collaborators discovered that in preparing microsome fractions, RNA occurred both in the fraction containing ribosomes bound to the endoplasmic reticulum and in the supernatant. They started referring to the RNA in the supernatant as "soluble RNA" or "S-RNA." Moreover, they found that labeled leucine was taken up by the S-RNA (Zamecnik et al., 1957; for an analysis of the discovery of soluble RNA, see Rheinberger, 1997). Smith, Cordes, and Schweet (1959) introduced the name "transfer RNA" (tRNA) for S-RNA, and proposed that it played a role in transferring the activated amino acid to the microsomal RNA, a suggestion Zamecnik readily adopted (Hoagland, Zamecnik, & Stephenson, 1959). The notion of transfer also suggests a role for this RNA in sequencing of amino acids, a suggestion that Zamecnik began to formulate:

> We have most lately been concerned with the possibility that at least a portion of the soluble RNA molecule to which the amino acid is attached is transferred along with the amino acid to the ribonucleoprotein particle, aligning itself in some base-pairing arrangement with the microsomal RNA prior to formation of a peptide chain. This concept agrees with the proposal of Crick that the soluble RNA molecule may serve as an adaptor in a base-pairing arrangement which determines amino acid sequence. (1958–9, p. 274)

As Zamecnik conceived the mechanism at this point, ribosomal RNA remained in place to direct multiple iterations of synthesis. The tRNA brought amino acids to the template as specified; the amino acids were added to the chain; and the tRNA departed. This made sense in eukaryote cells whose synthetic activities were limited to a few specific proteins. Research on bacteria generated a very different picture, for bacterial cells are capable of generating a wide range of proteins. Especially after the Pardee, Jacob, and Monod (1959) experiment showing the inducibility of protein synthesis, attention refocused on how temporary structures could be made from DNA, then move to the cytoplasm to direct protein synthesis (Brenner, Jacob, & Meselson, 1961). Investigations by Nirenberg and Matthaei (1961) that were directed toward developing a cell-free system for performing protein synthesis revealed that synthetic RNA created with uracil resulted in the synthesis of amino acid chains comprised of phenylalanine. Although this research is most celebrated for providing the first clue to the genetic code, it also provided compelling evidence for a third form of RNA, which came to be known as *messenger RNA* (*mRNA*), which was credited with carrying information about the protein to be synthesized from the nucleus to the ribosome.

The role of RNA in the ribosomes thus became less significant as attention turned to mRNA as the template and to tRNA as the transport for bringing amino acids to the template. This is ironic, because it was RNA that had been the distinguishing feature of first microsomes and then ribosomes. Research on the ribosomes themselves emphasized instead the proteins that comprised them. Those investigating the proteins in ribosomes deployed the same strategy as those investigating the mitochondrion: That is, they attempted to decompose the ribosome into different component parts. The particles separated in a centrifuge are often reported in terms of the time required for sedimentation, measured in terms of Svedberg units (one $S = 10^{-13}$ seconds); longer times correspond to lighter weights. Separating microsomes from yeast cells, Fu-Chuan Chao and Howard Schachmann (1956) reported 80S microsomes, which in turn dissociated into 60S and 40S units unless a trace of magnesium was present. (Svedberg units are not additive because the rate of sedimentation is affected by both the mass and shape of the particle.) Mary Petermann and her collaborators (Petermann et al., 1958) found that 78S liver ribosomes decomposed into 62S and 46S units. Finally, working with *Escherichia coli*, Alfred Tissiéres and James Watson (Tissiéres & Watson, 1958; Tissiéres et al., 1959) identified 70S ribosomes that separated into 50S and 30S units. From these studies, it appeared that ribosomes generally were comprised of two subunits of slightly different sizes, referred to as large and small subunits. Electron micrographs by Palade and his collaborators subsequently provided independent evidence for the two subunits of the ribosome (Sabatini, Tashiro, & Palade, 1966).

Research on the mechanism of protein synthesis involved not just the decomposition of the system into separate types of RNA and decomposition of the ribosome itself into subunits comprised of different forms of ribosomal RNA and protein, but also research on how these parts were organized. One important clue as to the organization of the parts stemmed from theoretical speculation about the relative size of messenger RNA molecules and ribosomes. Alexander Rich, a professor of biophysics at MIT, noted that the messenger RNA would be 1,500 Å or more in length, whereas ribosomes were only about 230 Å in diameter. Although messenger RNA chains might be wrapped around the ribosome, Rich concluded this was unlikely because then it would be difficult to maintain appropriate contact between messenger RNA and the ribosome. As Rich reported, "It occurred to us that proteins might actually be made on groups of ribosomes, linked together somehow by messenger RNA" (Rich, 1963, p. 45; see also Warner, Knopf, & Rich, 1963). As he described it, "the protein 'factories' of the cell are not single ribosomes working in isolation, but collections of ribosomes working together in orderly

fashion as if there were machines on an assembly line" (p. 44). Rich designated these groups *polyribosomes* or *polysomes*. To his characterization of the polysomes as constituting assembly lines, he quickly noted a difference between the ribosomal assembly line and human ones: "the polyribosome is not the usual kind of assembly line. In such an assembly line, the product moves down the line and component parts are added to it. In the polyribosome assembly line the ribosomes move down the line and each one makes a complete product."[34]

Evidence for polysomes came in two forms. The first were fractionation studies with rabbit reticulocytes, which are cells lacking a nucleus and specialized for manufacture of hemoglobin. Rather than using a medium of constant density, Rich centrifuged the contents in a solution spatially graded from 15% to 30% sucrose. Examination of the ultraviolet absorption characteristic of RNA in the centrifugation product revealed peaks in two fractions – one that corresponded to single ribosomes and the other to heavier materials, presumably polysomes. The amino acids in the preparation were labeled with C^{14}, making it possible to identify materials in which protein synthesis was occurring, and this showed a single peak corresponding to the polysome fraction. This suggested that the polysome was the locus of protein synthesis, a conclusion that was further supported by the fact that applying ribonuclease to the medium before centrifugation resulted in no fraction corresponding to polysomes and the radioactivity being transferred to the single ribosome fraction.

Rich collaborated with electron microscopists to develop a second form of evidence for polysomes. Using metal shadowing, the resulting electron micrographs clearly revealed clusters of ribosomes. In collaboration with Henry Slayter at MIT he used positive staining with uranyl acetate to develop micrographs that also revealed a thread 10 to 15 Å in diameter running between the ribosomes, which corresponds to the estimated thickness of a single strand of RNA. Calculating the diameter of the five polysomes attached to the thread and the gap between them resulted in a length of 1,500 Å, the expected length

[34] Rich seemed quite concerned with the appropriateness of the analogy. He returned to it again later in the paper and commented, "It is evident that protein synthesis is not really an assembly line process as it is normally understood. It would be more appropriate to compare protein synthesis with the operation of a tape-controlled machine tool. The tool will turn out an object of any shape within its range of capabilities, in response to information coded on the input tape. In factories where such tools are used each tool is provided with its own tape, but if it served any purpose a single tape could easily be fed through a battery of identical tools. The living cell evidently makes one tape serve for many tools because this is an efficient way to do the job" (pp. 50–1).

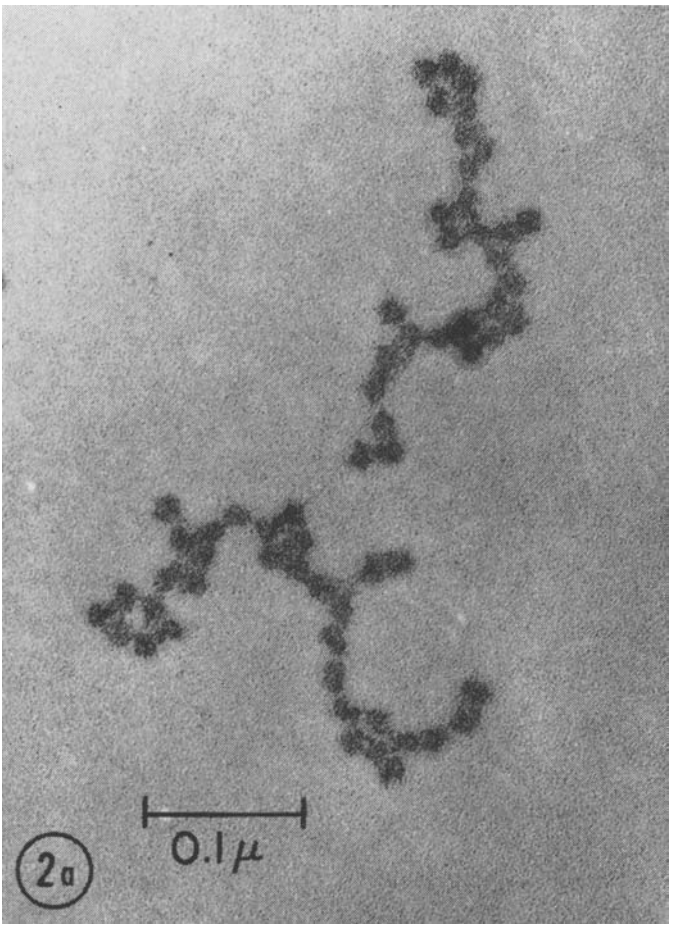

Figure 6.12. Electron micrograph of large polyribosomes from *E. coli* synthesizing β-galactosidase. Reproduced from H. Slayter et al. (1968), An electron microscopic study of large bacterial polyribosomes, *Journal of Cell Biology, 37*, 583–90, Figure 2a, p. 586, by copyright permission of the Rockefeller University Press.

of a strand of messenger RNA. Figure 6.12 shows a longer polysome from *E. coli* engaged in the synthesis of β-galactosidase (Slayter et al., 1968).

Rich proposed that the ribosomes moved along the messenger RNA, adding the appropriate amino acid to the polypeptide chain it was constructing according to the instruction at that locus on the chain. He hypothesized that a "ratchet-like mechanism" (1963, p. 49) would move the ribosomes along the chain. Thus, each ribosome attached to the messenger RNA made a copy of the

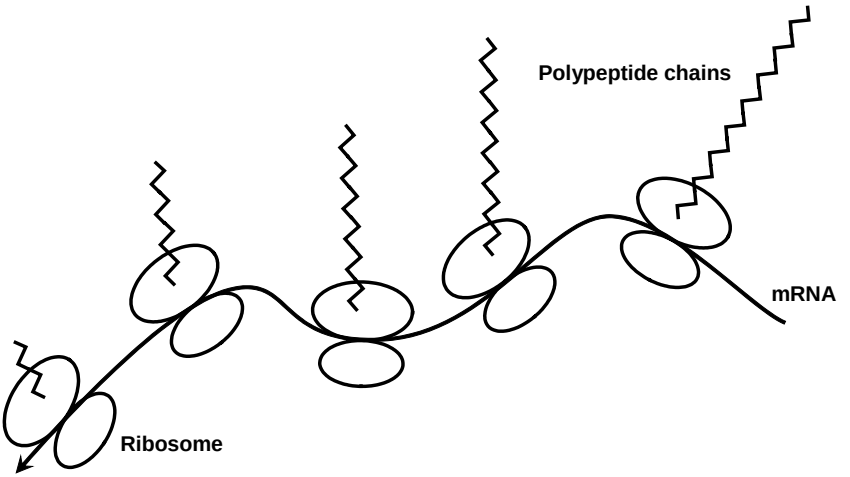

Figure 6.13. Schematic representation of a polyribosome. Five ribsosomes are attached to the mRNA. The leftmost ribosome has just been added to the mRNA string and is beginning to form a polypeptide chain, whereas the rightmost ribosome has already formed a fairly long chain.

same protein. As shown schematically in Figure 6.13, those far along the chain would have nearly completed polypeptide chains, and would drop off when they finished. New ribosomes would then join the chains and ribosomes early on the chain would have just the beginnings of the polypeptide chain completed.[35]

From the simple picture of the mid 1950s of one RNA-rich component of the cytoplasm serving as the locus of protein synthesis, a decade of research involving biochemists, cell biologists, biophysicists, geneticists, and molecular biologists had revealed a complicated structure of multiple component parts that performed different operations in the overall activity of protein synthesis. Although much remained to be filled in, a sketch of the mechanism at this lower level was established. There was no longer a mere empirical finding that ribosomes were involved in protein synthesis; rather, there was an account, in broad detail, of the mechanism responsible for protein synthesis.

[35] Rich also conducted a number of experiments to test his model of the polysome assembly process. First he utilized a homogenizer before centrifugation, which produced a number of peaks in terms of both ultraviolet absorption and radioactivity. He proposed that each successive peak corresponded to an additional ribosome in a cluster, a proposal he confirmed in collaboration with electron microscopist Cecil Hall by subjecting material from each fraction to electron microscopy and finding evidence of the predicted clusters.

Transporting Newly Sequenced Polypeptides

Although cell biologists' tools of electron microscopy and cell fractionation played important roles in unraveling the mechanism through which different types of RNA contributed to protein synthesis, cell biologists were also interested in the fate of newly formed proteins. Utilizing C^{14} labeling in pancreatic microsomes from pigeons, Colvin Redman, Siekevitz, and Palade (1966) found evidence that labeled amylase, the secretory protein being synthesized, would appear in the cisternal cavities of the microsome. Subsequent research by Redman and David Sabatini (1966) demonstrated that treating the ribosome with puromycin resulted in the appearance of labeled unfinished proteins in the cisternal cavities. They viewed this as showing that "from the onset of protein synthesis the growing peptide chain is directed towards the cisternal space into which it diffuses upon its release from the attached ribosome" (p. 608). Earlier research by Sabatini (Sabatini et al., 1966) had demonstrated that it was the large ribosomal subunit that was directly attached to the membrane of the endoplasmic reticulum, leading to the conclusion that the new protein was transported from the large subunit through the membrane to the cisternal space. Palade, in his 1974 Nobel Lecture, noted that the vectorial transport of newly formed proteins into the cisternal space provided the only known explanation for the complex structure of the endoplasmic reticulum:

> This conclusion provides a satisfactory explanation for the basic structural features of the endoplasmic reticulum: a cavitary cell organ of complicated geometry which endows it with a large surface. All these features make sense if we assume that one of the main functions of the system is the trapping of proteins produced for export. With the exception of Ca^{2+} accumulation in the sarcoplasmic reticulum, i.e., the equivalent cell organ of muscle fibers, no other recognized function of the endoplasmic reticulum (e.g., phosphatide and triacylglycerol synthesis, mixed function oxygenation, fatty acid desaturation) requires compellingly and directly a cavitary organ, at least according to our current knowledge. (Palade, 1992, p. 183)

As we will see, the next stage in the movement of the newly formed proteins was to the Golgi apparatus, and this discovery led Palade to reverse his earlier denial of the reality of the Golgi and to conduct landmark studies on its function.

3. TWO ADDITIONAL ORGANELLES

Research in the 1950s and early 1960s on the mitochondrion and the endoplasmic reticulum resulted in the first mechanistic models of cellular functions.

In each case, combining morphological and biochemical inquiry resulted in differentiation of component parts and proposals as to the specific operations performed by those parts. Another development over that period, though, was the discovery that yet other cell organelles performed different functions that contributed to the overall life of the cell. Particularly important in this respect was research on the Golgi apparatus and the lysosome.

The Golgi Apparatus

As I discussed in Chapter 4, in 1949 Claude and Palade challenged the existence of the Golgi apparatus, contending that it was an artifact of staining with osmium or other heavy metals. Claude and Palade's challenge to the reality of the Golgi apparatus was one of the last. A prominent exception was a charge by John Baker (1957; 1963) that many different substances were being conflated under the one label. As I argued in Chapter 3, the Golgi apparatus was a natural target for charges of artifact since, up through Claude and Palade's challenge, observations of it were generally limited to cells fixed with osmium compounds and the evidence for its functional role was far from overwhelming. But this situation changed so definitely that, when Palade together with Marilyn Farquhar (Farquhar & Palade, 1981) wrote a review of research on the Golgi apparatus they commented that "now no one questions that the Golgi apparatus is a distinct cell organelle, or is unaware of its participation in a wide variety of cellular activities. Indeed, the Golgi apparatus, or Golgi complex as it is often called, not only occupies the cell center, but it also has moved toward center stage, because it has been shown to be involved in so many cell activities" (p. 77s). Interestingly, given his earlier opposition, Palade contributed much to the vindication of the Golgi apparatus by providing crucial information about its function.[36]

[36] Despite the vehemence of the challenge he and Claude had issued, in the review of the history of the Golgi apparatus with Farquhar, Palade does not mention his own role as one of the last to challenge the reality of the Golgi apparatus. Farquhar and Palade (1981) write, "The period before the mid 1950s was characterized by controversy concerning the reality of the Golgi apparatus, with the scientific community divided into nonbelievers and believers. The acceptance of the status of the Golgi as a bona fide cell structure depended on whether one believed that the metallic impregnation methods (involving use of silver or OsO_4), which Golgi and others used to demonstrate the apparatus, were staining a common structure with variable form and distribution in different cell types, or alternatively, that these methods resulted in artifactual deposition of heavy metals on different cell structures in different cell types" (p. 77s). Again in 1998, on the occasion of the 100th anniversary of Golgi's discovery, Farquhar and Palade make no reference to Palade's role as challenger of the Golgi: "The debate raged because the Golgi was not visible in living cells and its visualization depended on Golgi's capricious heavy-metal staining method, called the black reaction (la reazione nera), which was difficult to reproduce reliably and stained many other structures, including whole neurons" (p. 2).

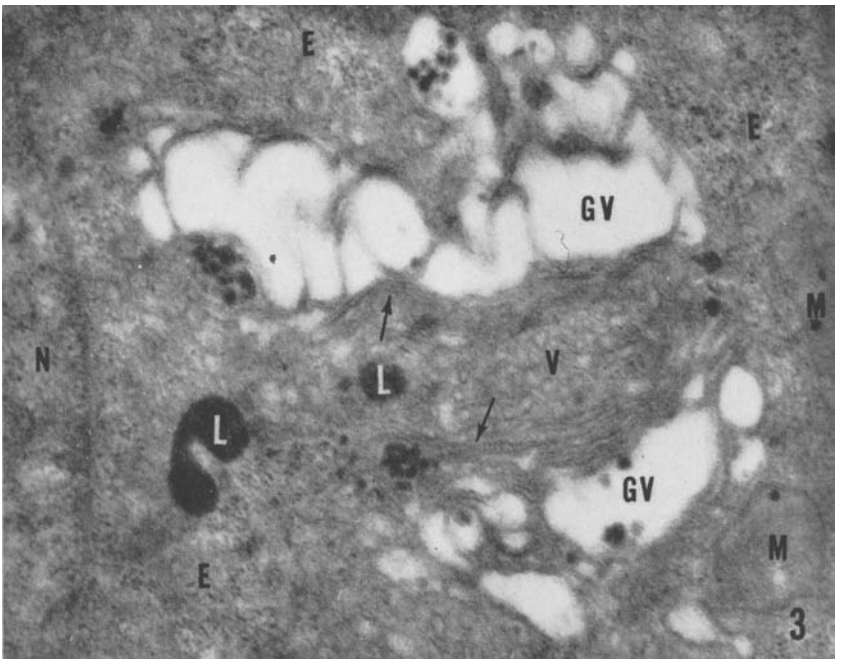

Figure 6.14. Electron micrograph of Golgi complex in a principal cell of the duodenum of a mouse. The arrows mark the Golgi membrane (Golgi lamellae or flattened cisternal sacs). Also indicated are GV: Golgi vacuoles; V: Golgi vesicles; L: Lipid droplets; M: mitochondria; N: nucleus; and E: ergastoplasm (endoplasmic reticulum). Reproduced from A. J. Dalton and M. D. Felix (1956), A comparative study of the Golgi complex, *Journal of Biophysical and Biochemical Cytology, 2* (No. 4, Part 2), 79–84, Figure 3, plate 27, by copyright permission of the Rockefeller University Press.

Before Palade returned to studying the Golgi apparatus, electron microscopy, especially studies by Albert Dalton, played a major role in providing additional information about its structure. In an initial study, Dalton (1951b) found no evidence of the formation of myelin forms when hepatic and intestinal epithelial cells were fixed with Champy's fluid. Nonetheless, a membranous network was visible in electron micrographs in the parts of cells where the Golgi apparatus had typically been detected, thereby undercutting Palade and Claude's proposal as to how the Golgi arose as an artifact.[37] In several subsequent studies with Marie Felix, Dalton uncovered the detailed structure

[37] Dalton's subsequent research with Felix further challenged Palade and Claude's proposal. By examining the process of fixation, they determined that the Golgi material responded very differently than lipid droplets, thereby dispelling Palade and Claude's contention that the Golgi apparatus was an artifact produced from such lipid droplets.

of the Golgi apparatus (Dalton & Felix, 1954; Dalton & Felix, 1955; Dalton & Felix, 1956). As illustrated in Figure 6.14, they differentiated three components – a system of lamellae or flattened cisternal sacs, large vacuoles, and clusters of small vesicles. The flattened sacs (saccules) typically occur in stacks of three to seven in plant and animal tissues, and in larger numbers in unicellular organisms. The saccules are the major contributor to the traditional image of the Golgi apparatus in light microscopy. On one side, the *cis* side, the stacks abut the endoplasmic reticulum. Clusters of small vesicles are visible at the interface between the endoplasmic reticulum and the *cis* side of the stack. On the other side, the *trans* side, of the stack, larger vacuoles are found that are often referred to as condensing vacuoles. To highlight the fact that there are several different components comprising the Golgi region, Dalton introduced the term *Golgi complex*. In addition to providing much better images of the Golgi apparatus than had been available through light microscopy, Dalton and Felix also sought to address its functional significance. By comparing the images of the Golgi complex in mouse intestinal cells after fasting and forty minutes after eating, Dalton and Felix provided evidence that the Golgi apparatus is involved in storage of lipids after absorption.

Other early electron micrographs of the Golgi apparatus (Sjöstrand & Hanzon, 1954; Farquhar & Rinehart, 1954) revealed a close relation between secretory granules and the Golgi apparatus. Subsequent micrographs seemed to show secretory granules within the Golgi structures:

> Secretory granules have been seen within components of the Golgi bodies of rat pituitary acidophils and mouse pancreatic acinar cells. The fact that secretory granules are much more frequently encountered within Golgi components under conditions of increased secretory activity suggests that granule formation may occur within the Golgi apparatus in these two types of cells. (Farquhar & Wellings, 1957, p. 321)

Applying biochemical analysis required separating the Golgi apparatus from other cell components through cell fractionation. In addition to his electron microscopy of the Golgi apparatus, Dalton (Dalton & Felix, 1954) established that the Golgi apparatus could be extracted from epididymis cells, and Schneider and Kuff (1954) separated it within epididymal homogenates by gradient centrifugation. Schneider and Kuff produced evidence that the Golgi fraction was comprised of high concentrations of phospholipid as well as acid and alkaline phosphatase and RNA. However, fractionation of the Golgi apparatus was more challenging than fractionation of other organelles. Its smooth lipoprotein membranes, for instance, tended to break up and form smooth microsomes (Whaley, 1975, p. 31). A decade later Morré and his

collaborators developed improved procedures for subfractionation of Golgi fractions, and Fleischer, Fleischer, and Ozawa (1969) localized galactosyltransferase in the Golgi fraction – the first enzyme to be primarily associated with the Golgi apparatus.

After contending that the Golgi apparatus was an artifact of osmium staining, and prohibiting discussion of it in the Rockefeller laboratory for a number of years, George Palade was led back to investigating the Golgi apparatus in the 1960s as an outgrowth of his biochemical research on protein synthesis in ribosomes discussed previously. Palade and Siekevitz, using ^{14}C-leucine as a tracer, established a migration in the pancreatic exocrine cell of α-chymotrypsinogen from the ribosome into the lumen of the endoplasmic reticulum and ultimately into zymogen granules which were excreted from the cell. Detailing the path and activities occurring during this migration now became a focus of research. In an initial study in Palade's laboratory, Lucien Caro (1961; see also Caro & Palade, 1964) used ^{3}H-leucine, which appeared within three to five minutes of injection in the endoplasmic reticulum, and then after twenty to forty minutes in the condensing vacuoles on the *trans* side of the Golgi stack.[38] After an hour the label appeared in the zymogen granules. This was critical evidence in establishing the transport of secretory proteins from the ribosomes through the membranous system of the Golgi apparatus to the zymogen granules that would then be secreted.

These initial findings were further elaborated in a series of studies with graduate student James Jamieson employing tissue slices from guinea pigs beginning in 1966. They arrived at a more detailed characterization of the migration of membrane-bound vesicles from the endoplasmic reticulum through the components of the Golgi apparatus to discharge from the cell (Jamieson & Palade, 1966; Jamieson & Palade, 1967a; Jamieson & Palade, 1967b). Key to their work was the use of three-minute exposure to leucine-^{14}C, a radioactive amino acid ("labeling pulse"), followed by removal of the unincorporated label ("chase"), allowing for better time resolution of the radioactive material.

Jamieson and Palade demonstrated that proteins, after leaving the endoplasmic reticulum, are encapsulated in small peripheral vesicles on the *cis* side of the Golgi stack and appear (after about thirty minutes) in condensing vacuoles on the *trans* side of the stack. In the interval they presumably traveled through the Golgi stacks, although Jamieson and Palade do not focus on that

[38] At about the same time, Warshawsky, Leblond, and Droz (1961) used labeled leucine in pancreas cells to trace the sites of uptake and the path of migration. They, however, lacked the ability to examine the results with the electron microscope.

period. After the vacuoles reach the *trans* side of the stack, they migrate to the cell membrane, where the membrane of the vacuole merges with the cell membrane. There is then an opening of the joined membranes so that the contents of the vacuoles are released into the extracellular matrix without breaching the diffusion barrier provided by the cell membrane. Palade (1958b) originally termed this process "membrane fusion," but it was relabeled "exocytosis" by de Duve (1959).

Beyond tracing the course of protein transport, Jamieson and Palade set out to determine how tightly the processes of protein synthesis and transport are coupled and to identify the energy source for the process. Using cyclohexamide to block protein synthesis, Jamieson and Palade (1968a) were able to uncouple the synthesis of protein from its transport to the Golgi complex, showing that transport did not depend on new proteins entering the process. In a subsequent paper (Jamieson & Palade, 1968b), they addressed the energy requirements for the process by demonstrating that the glycolytic inhibitors (fluoride, iodoacetate) failed to block transport, but that respiratory inhibitors (N2, cyanide, antimycin A) and inhibitors of oxidative phosphorylation (dinitrophenol, oligomycin) did. Jamieson and Palade then speculated about what operation required energy:

> At present, it is clear that the energy is used to connect the RER cisternal space with that of the condensing vacuoles, and that the small peripheral vesicles of the Golgi complex participate in the connection. The details of this operation, however, remain obscure: the cell may establish intermittent connections between these two compartments or effect transport between them using the small peripheral vesicles as shuttle carriers. Both alternatives imply repeated membrane fission-fusion and this is most probably the energy-requiring event. (p. 599)

Jamieson and Palade clearly favored the hypothesis that small peripheral vesicles serve as shuttle carriers and that the nascent proteins remain membrane bound as they transverse the Golgi structure. Prior to their research, P. P. Grassé (1957), relying on early electron micrographs, had proposed a maturational or cisternal progression model according to which cisternae were continually being created on the *cis* side of the stack and matured as they moved to the *trans* side, where they disintegrated and released the newly synthesized products for further transport. Neutra and Leblond continued to espouse this view (Neutra & Leblond, 1969, p. 105), but in subsequent years evidence built for Palade's proposal and it became the dominate view.

Palade's early research did not focus on the Golgi stack, and to the degree that he investigated the function of the Golgi components, he focused on

concentrating proteins in secretory granules. What did the stack, the most prominent component of the Golgi apparatus, contribute? Marian Neutra and Charles Leblond (1969, p. 103) posed the question:

> Why do these proteins pass through the Golgi apparatus? Do they undergo some essential processing operation there? One can see with the electron microscope that proteins come out of the apparatus neatly packaged in globules whose membranes have been donated by the Golgi saccules. It seems hardly likely, however, that this elaborate system exists simply for the purpose of putting the proteins in bags; nature has a way of avoiding complex solutions for simple problems. We therefore decided that a closer look had to be taken at the protein products themselves to determine if their sojourn in the Golgi apparatus was responsible for some important change in their chemical form.

An important clue was that most secretory products are not proteins alone, but rather proteins linked to carbohydrates. Leblond and his collaborators at McGill University studied the goblet cell of the colon, which secretes such a compound – mucigen. These goblet cells are long, narrow structures squeezed between other cells in the intestinal lining. Each contains several Golgi stacks of eight to ten saccules. The bottom or *cis* saccules are flattened in appearance, whereas the top or *trans* saccules are bulging with material. Above them are mucus globules that are excreted in due course. In autoradiographic studies with Marian Peterson, Leblond showed that glucose tracer first appeared in the cisternae of the Golgi apparatus five to fifteen minutes after injection and moved progressively to the more distal cisternae, with the distal cisterna being converted into mucigen granules (Peterson & Leblond, 1964a; Peterson & Leblond, 1964b). Subsequently, Neutra and Leblond (1966a; 1966b) proposed that the glucose or galactose precursors enter the goblet cell from a capillary and move directly to the Golgi apparatus, where glucose is combined with proteins synthesized in the endoplasmic reticulum to form glycoproteins. As Neutra and Leblond (1969) related, by the end of the 1960s evidence had accumulated that the Golgi apparatus performed a variety of roles in the construction of large carbohydrate molecules such as adding sulphate bonds to create polysaccharide secretion products.

After decades during which it was suspected to be an artifact, in the 1960s the Golgi apparatus came to be generally recognized as a major component of the machinery of the cell. In part its rehabilitation stemmed from the ability of the electron microscope to provide more detailed images of its structure, but of even greater significance was the development of an account of its functional significance in the generation of products that figured in cell secretions processes.

The Lysosome

The lysosome is the one cell organelle that came to prominence in the early years of cell biology that had no direct roots in classical cytology.[39] Rather, Christian de Duve discovered it in the course of research he began in 1949, when he assumed directorship of what he characterized as "the derelict laboratory associated with the Chair of Physiology at the University of Louvain," directed at isolating glucose-6-phosphatase in liver through differential centrifugation. He became interested in glucose-6-phosphatase the previous year, which he spent with Carl and Gerty Cori at Washington University.[40] The Coris had been investigating glucose metabolism and had discovered in liver a hexose phosphatase. Working with liver extracts, de Duve identified the hexose phosphatase as a specific glucose-6-phosphatase and differentiated it from acid phosphatase. When he precipitated the glucose-6-phosophatase in an acid solution, he found he could not redissolve it when he raised the pH level. De Duve had learned from Claude[41] that his large fraction would agglutinate at acid pHs. This suggested to de Duve that agglutination, not precipitation, was occurring in his glucose-6-phosphatase preparations – the enzyme was attached to a structure. He then turned to cell fractionation (helped in part by Claude, who had moved back to Belgium after leaving the Rockefeller Institute) as a gentler way of separating the enzyme than the Waring blender that he had been using, and localized 95% of its activity in the microsomal fraction. What caught de Duve's attention, though, was the fact that the homogenate he prepared before fractionation exhibited only 10% of the acid

[39] In part this is due to the fact it was first identified through its function, not its cytological structure, which Novikoff (1970, p. 121) identified as unusual: "Historically, study of organelles begins usually with the accumulation of morphological observations and then passes to the isolation of the organelle in relatively pure fraction and biochemical study. For lysosomes, however, this pattern was reversed."

[40] Before going to the Coris' laboratory, de Duve had spent eighteen months in Hugo Theorell's laboratory in Sweden where he mastered biochemical techniques. He was interested in visiting the Coris because his earlier research indicated that they had incorrectly ascribed to insulin actions that were not due to insulin itself but to glucagon, a contaminant in their preparations. Carl Cori initially rejected de Duve's request to spend six months in their laboratory but shortly thereafter Earl Sutherland, a postdoctoral fellow in the Coris' laboratory (who later won a Nobel Prize for the discovery of cyclic AMP), obtained evidence that glucagon was the responsible agent. Cori invited de Duve to come and collaborate with Sutherland, which de Duve was able to do with support from the Rockefeller Foundation. The identification of impurity in insulin also paid off handsomely for de Duve. He had determined that insulin prepared by Eli Lilly bore the glucagon contaminant and for alerting them to this fact, the company provided de Duve's laboratory at Louvain with a $5,000/year research budget (Interview with Christian de Duve, 5 December 1995, Rockefeller University, New York).

[41] On his return from St. Louis, de Duve had stopped to visit Claude at the Rockefeller Institute; he reports reading several of Claude's papers on the flight back from New York.

phosphatase activity found in homogenates prepared with the Waring blender. (The activity in the fractions isolated by centrifugation was higher but still lower than in Waring blender preparations.) Convinced that this represented an error in the assay, he stored the fractions in a refrigerator. When he repeated the assay five days later it was unexpectedly an order of magnitude greater in all fractions and in the range expected from Waring blender preparations in the mitochondrial fraction.[42] He concluded that the activity of the enzyme must have been masked in the fresh preparation and only activated over time.

Soon de Duve pursued the latency in the enzyme activation and proposed that upon initial fractionation the acid phosphatase was contained within a separate "baglike" particle that limited its access to the substrate. The rough treatment in the Waring blender or the gradual aging of the homogenate prepared for cell fractionation released the enzyme from this container. Only once released was it possible to assay its activity. This readily explained why acid phosphatase did not destroy the various phosphate compounds found elsewhere in the living cell. The fact that, with the four-fraction technique, aged mitochondrial fractions yielded the highest levels of acid phosphatase activity suggested that the mitochondrion itself was the sac housing the digestive enzymes (Berthet & de Duve, 1951). De Duve discovered that it was not the mitochondrion when the high-speed attachment to de Duve's centrifuge broke and François Appelmans, a medical student working with him, had to prepare mitochondria using an ordinary preparatory centrifuge with longer centrifugation times. The resulting mitochondria showed no acid phosphatase activity. This led de Duve to continue fractionation, segregating a light fraction containing acid phosphatase and a heavy fraction containing cytochrome oxidase (Berthet et al., 1951; de Duve & Berthet, 1954).

At the Second International Congress of Biochemistry in Paris in 1952, de Duve presented his claim that acid phosphatase belonged to a special cytoplasmic particle. Afterward, P. G. Walker, a British biochemist, related to him that he had found similar results with β-glucuronidase (Walker, 1952); de Duve then tested his light fraction and discovered that it contained β-glucuronidase as well. Subsequently he investigated several other enzymes – acid ribonuclease, acid deoxyribonuclease, cathepsin, urate

[42] In a historical rendition of the events, de Duve (1969, p. 7) wrote, "we could have rested satisfied with this result, dismissing the first series of assays as being due to one of those troublesome gremlins that so often infest laboratories, especially late at night . . . Two factors saved us. . . . The assays had been repeated with the old as well as with fresh reagents, giving identical results. The gremlin, if he was the culprit, must have been a very subtle one. Furthermore, we had noted that the greatest discrepancy between the two series of results occurred in the mitochondrial fractions, the smallest one in the supernatant fraction."

oxidase, NADH cytochrome *c* reductase, NADPH cytochrome *c* reductase, and fumarase. Although the cytochrome *c* reductases and fumarase did not, acid ribonuclease, acid deoxyribonuclease, cathepsin all fractionated with acid phosphatase and β-glucuronidase. Each of the enzymes that fractionated together functions, in conjunction with water, to degrade a macromolecule into its subunits through a reaction that follows the formula

$$AB + H_2O \rightarrow AH + BOH.$$

The hydrolytic nature of these enzymes led de Duve to propose the name *lysosome* (from the Greek work *lusis*, meaning to untie) for this component (de Duve et al., 1955).[43] He also proposed that there was a good reason why this group of enzymes might co-occur in a separate organelle: otherwise they would interfere with synthetic processes and disrupt cell structure. Urate oxidase had a similar distribution as the lysosome enzymes but showed no latency and was not brought into solution by the same treatments as sufficed for the hydrolases. In subsequent research, he identified it as a constituent of yet another organelle, the peroxisome.[44]

In conjunction with the Third International Congress of Biochemistry in Louvain in 1955 Alex Novikoff visited de Duve's laboratory for six weeks. Novikoff took samples of de Duve's lysosome preparation to Claude's laboratory in Brussels and then to Bernhard's laboratory in Paris to examine them with the electron microscope. The micrographs revealed particles[45] that had occasionally been seen in electron micrographs a year earlier by Charles Rouillier, who had named them "pericanalicular dense bodies" because they were structures impenetrable to electrons found along bile canaliculi. Novikoff described these structures as having a mean length of 0.37μ and as

[43] The choice of name was explained by de Duve (1969, p. 14): "*Lysosome* sounded too much like *lysozyme*; *lysosome* could be confused with *lyo-enzyme*, which already had a meaning; *hydrosome* brought to mind the image of some marine contraption. We finally settled for *lysosome*, well aware of the danger of our choice." By the early 1960s a total of twelve enyzmes were associated with the lysosome, each capable of splitting important biological compounds in a slightly acid environment: acid phosphatase, cathepsin A and B, acid desoxyribonuclease, acid ribonuclease, β-glucuronidase, arhylsulfatase A and B, phosphoprotein phosphatase, β-galactosidase, β-N-acetylglucosamidase, and α-mannosidase (Novikoff, 1961). By 1980 the number had grown to thirty-six.

[44] In these investigations, de Duve found that urate oxidase segregated with three additional enzymes, two of which were involved in the synthesis of hydrogen peroxide (d-amino acid oxidase and α-hydroxyl acid oxidase) and one in its breakdown (catalase). He linked all four enzymes to the peroxisome, which he identified with what had previously been referred to as *microbodies*.

[45] His experience of seeing the micrographs was described by de Duve (1969, p. 16) as "like Le Verrier after the planet Neptune was discovered."

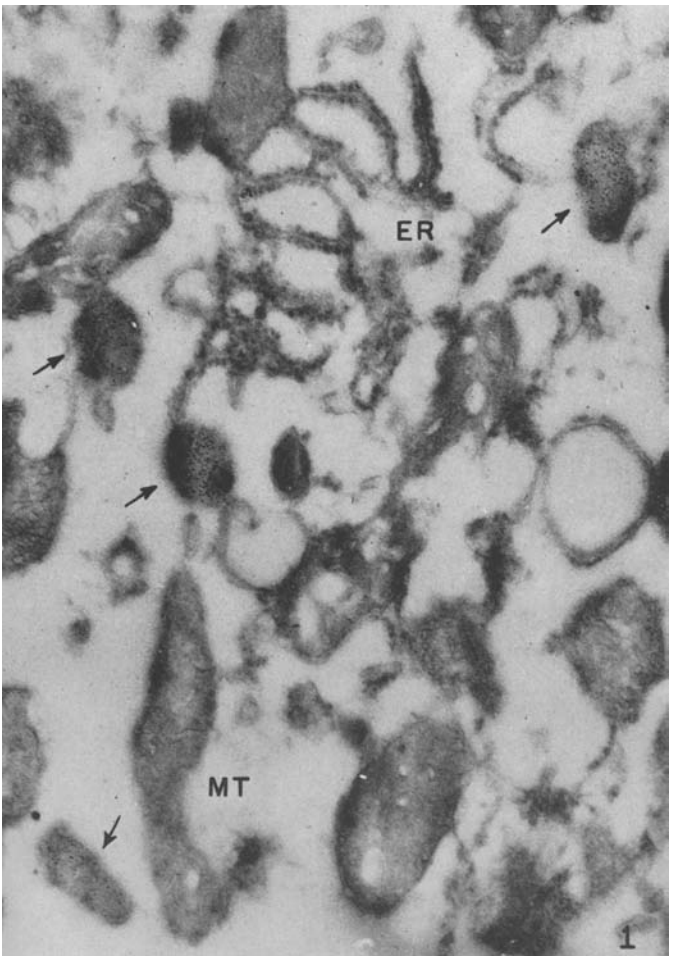

Figure 6.15. Electron micrograph of lysosome fraction from rat liver. Arrows indicate the dense bodies taken to be the lysosomes. ER designates microsomal membranes, presumably from the endoplasmic reticulum; the granules within them are much smaller than the dense bodies (lysosomes). MT designates a mitochondrion, which the authors note is not well-preserved. Reproduced from A. B. Novikoff, H. Beaufay, and C. de Duve (1956), Electron microscopy of lysosome-rich fractions from rat liver, *Journal of Biophysical and Biochemical Cytology, 2* (No. 4, Part 2), 179–84, Figure 1, plate 60, by copyright permission of the Rockefeller University Press.

containing tiny, electron-dense granules and sometimes possessing internal cavities and external membranes (Figure 6.15). They tentatively identified the particles as the lysosomes (Novikoff, Beaufay, & de Duve, 1956). Novikoff (1961) went on to develop light and electron microscope stains for acid phosphatase and used the presence of a membrane and the positive indication of acid phosphatase as evidence for lysosomes, noting that they often differed substantially in size in different cell types.[46] Treating intracellular digestion as the defining function of lysosomes, Novikoff proposed differentiating lysosomes according to the types of exogenous or endogenous material the lysosome would digest.

Working at the State University of New York College of Medicine in Brooklyn during the same period as de Duve, Werner Straus identified droplets in the proximal tubule of the kidney that stored and broke down reabsorbed proteins. He determined that they possessed an unusually high concentration of acid phosphatase (Straus, 1954). After learning of the other enzymes de Duve associated with lysosomes, Straus (1956) determined that they were present in his droplets as well. Straus's research drew attention to the connection between lysosomes and the digestion of material brought into the cell as well as breakdown products of the cell itself. This idea of the function of the lysosome was further developed by de Duve (1958):

> Our working hypothesis will therefore be that lysosomes are involved in processes of acid hydrolysis. These may comprise: digestion of foreign material, engulfed by pinocytosis, athrocytosis or phagocytosis; physiologic autolysis, as presumably occurs to some extent in all tissues, and particularly as part of the more specialized processes of involution, metamorphosis, holocrine secretion, etc; pathological autolysis or necrosis. (p. 146)

Initially it was difficult to identify lysosomes in other cells due to the variability in their shape and size, causing de Duve to be reluctant to generalize the lysosome concept. Once their role in cellular digestion was understood, this variability made sense: "This polymorphism of the lysosomes is now perfectly understandable: their digestive activity causes them to be filled with a variety of substances and objects in an advanced state of disintegration,

[46] Regarding this, de Duve (1969, p. 16) commented, "I must confess that Novikoff, who pioneered this field with untiring energy, received little encouragement on my part. I objected strongly to what I considered a misappropriation of the word *lysosome*, which in my own copyrighted version implied the simultaneous presence of several acid hydrolases, and which he was now using to designate any structure giving a positive reaction for a single such enzyme." Novikoff, however, expressly agreed with de Duve that *intracellular digestion* was the defining function of lysosomes (Novikoff & Holtzman, 1970).

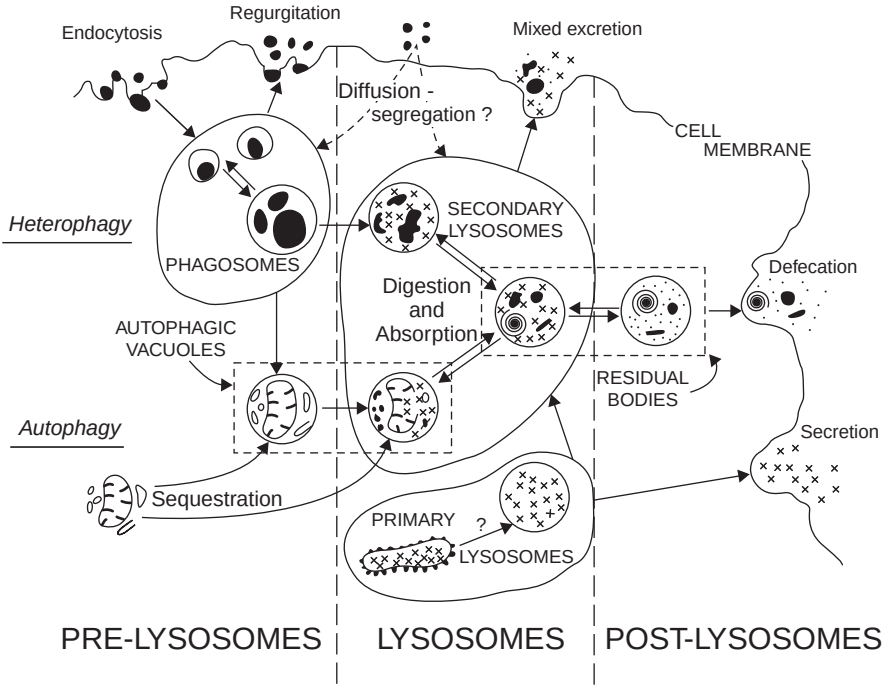

Figure 6.16. Schematic representation by de Duve and Wattriaux (1966) of the lysosomal system for eliminating cellular waste products. There are variations for material entering the cell from without (heterophagy) and from within (autophagy), both having three phases. On the left side, denoted *pre-lysosomes*, the incorporation of either endocytic vesicles into phagosomes or old cell parts into autophagic vesicles is illustrated. In the middle portion, these vesicles are shown as merging with primary lysosomes, generating secondary lysosomes. The crosses symbolize hydrolases. Finally, on the right side the packaging of the undigested material into residual bodies is illustrated. Reprinted, with permission, from the *Annual Review of Physiology*, *28*, Figure 6, p. 468, ©1966 by Annual Reviews, www.annualreviews.org.

and it is their contents that determine their shape, size, density and so on" (de Duve, 1963, p. 76).

Typically, the development of an understanding of how a mechanism works requires decomposing a system to determine the various component parts and their operations. In the case of the lysosome, the discovery that it was a sac containing hydrolytic enzymes made it clear how the key component part of the mechanism worked. What was required was to understand how it interacted with other components to perform the function of digesting and recycling both old cell components and material that had been brought into the cell. Developing the account of the mechanism thus required relating this

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component part and its operation to other parts and their operations. In the early 1960s de Duve and others succeeded in piecing together such an account of the lysosomal system (Bainton, 1981).

As shown in Figure 6.16, de Duve presented parallel versions of the mechanism for the digestion of material entering the cell from without (heterophagy) and from within (autophagy). In heterophagy, the material to be digested by the lysosomal enzymes was first entrapped into what he called a *phagosome*. He proposed these vesicles then fused with the lysosome sac, which he called the *primary lysosome*, creating a digestive vacuole or *secondary lysosome*. Some of the digestion products diffused back into the cytoplasm of the cell while materials resistant to attack built up in the vacuole, creating what he called a *residual body*, which either was expelled or continued to build up in the cell. In autophagy, de Duve called the digestive vacuole an *autophagic vacuole*; these were operated on in the same manner as phagosomes. He developed a technique for staining for the activity of acid phosphatase, using lead to generate an insoluble compound that has a high electron scattering potential. This yielded a dark image in the micrographs that enabled visualization not only of the lysosome itself but also of the digestive vacuole, autophagic vacuole, and residual body. Inside the autophagic vacuoles, it was possible to recognize remnants of mitochondria and the endoplasmic reticulum (de Duve, 1963).

A last piece of the story of the lysosome is an account of its formation in the cell. Because hydrolytic activity is the defining mark of the lysosome, the discovery of acid phosphatase also in some cisternae of the *trans* region of the Golgi apparatus and in adjacent smooth endoplasmic reticulum led Novikoff and his colleagues to designate the area GERL (a Golgi-related region of smooth endoplasmic reticulum from which lysosomes appear to develop) (Holtzman, Novikoff, & Villaverdi, 1967). Novikoff advanced the idea that hydrolases bypass the Golgi stack and are transported directly to the most distal area of the Golgi apparatus for incorporation into primary lysosomes.

Research on the lysosome by de Duve encapsulates the productive coordination of the results of structural and functional decomposition in discovering cell mechanisms. Modifying the techniques of cell fractionation, he identified a new fraction whose contents indicated their function. Moreover, their operation turned out to require isolation if they were not to destroy the cell itself. Comparing electron micrographs of the fraction with micrographs of cells revealed the locus of the organelle in the cell. At this stage the lysosome had a structural identity and a function, but understanding its operation required postulating other components, whose existence could also be identified in

micrographs. The outcome of the coordinated work on structure and function was an ingenious account of the lysosome mechanism.

4. CONCLUSION

By the 1960s the efforts begun in the 1940s bore fruit in the articulation of a number of mechanisms operative in the cytoplasm of the cell. Understanding these mechanisms required the collaborative effort of morphologists and more functionally oriented investigators. Two of these efforts followed up on the initial forays at Rockefeller and elsewhere in the 1940s in identifying the mitochondrion as the power plant of the cell and identifying the microsomes and what came to be known as the endoplasmic reticulum as new components of the cytoplasm. In the case of the mitochondrion, these efforts led not only to an understanding of why the enzyme systems responsible for cellular respiration always involved a membrane component but also of how the membrane played a crucial role in the actual functioning of the mechanism. In the case of the endoplasmic reticulum, researchers not only identified the major steps in which the ribosomes and other RNA constituents operate to synthesize proteins, but also determined the role of the membranes in structuring environments for the newly synthesized proteins destined for export. Investigations in the 1950s and 1960s also finally established the reality and function of the Golgi apparatus, which had been the focus of bitter battles during the first half of the twentieth century. By discovering the process of migration from the rough endoplasmic reticulum through the smooth endoplasmic reticulum into the Golgi region and then into secretory vesicles, investigators came to recognize the Golgi apparatus as playing crucial roles in the preparation of proteins for export. And finally the discovery of the lysosome as a new organelle rich in hydrolytic enzymes provided the basis for developing the basic account of how cells digested either foreign substances brought into the cell or worn out cell components. By the 1960s cell biologists recognized that the cytoplasm was rich in mechanisms that play critical roles in the maintenance of cell life and understood the major parts and operations in each mechanism.